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ANNOUNCEMENTS

Gastrointestinal Cancer Conference

The Proceedings of the Sixth National Gastrointestinal Cancer Conference, sponsored by the National Cancer Institute, and held in New York, April 1955, appear in the October issue of Gastroenterology. Proceedings of the previous five Conferences can be found in the Journal of the National Cancer Institute, volume 1, page 423; volume 5, page 311; volume 7, page 299; volume 10, page 297; and volume 13, page 927.

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Virus of Avian Erythromyeloblastic Leukosis. IX. Antigenic Constitution and Immunologic Characterization [1, 2]

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Introduction

In 1949, studies were undertaken in this laboratory on the virus of one form of avian erythromyeloblastic leukosis. It has been concluded that the condition under study is that which is designated as *myeloblastosis* (*1*) (see p. 595 for further discussion). The objective of the work was isolation of the agent in the state of homogeneity and in amounts compatible with quantitative physical, chemical, and biological studies. Earlier work by other investigators (*2*, *3*) had shown that the plasmas from chickens with the disease contained the infectious entity, though in unknown physical amounts. Examination of such plasmas after filtration (*4*) corroborated the previous findings and, in addition, revealed the presence of a particulate material (*5*, *6*) as shown by electron microscopy. The particles occurred in some plasmas in very large numbers (*7*), and because they were specific to the diseased chickens, the particles were judged to be the virus. This view was strengthened by the observation that little material extraneous to the characteristic particles could be seen in electron micrographs obtained directly with the plasma or with ultracentrifugal concentrates of the particles. There seemed no reason at this stage to doubt that the virus constituted an essentially homogeneous population of particles suspended in the solution of plasma proteins. It appeared, therefore, that purification should be a matter simply of repeated sedimentation of the particles in order to wash away the proteins of low molecular weight. With this assumption, numerous experiments were made by the usual methods of ultracentrifugal fractionation to obtain the virus particles in purified preparations. The results of various physical studies by electron microscopy, analytical ultracentrifugation, and other procedures provided much evidence of the homogeneity of the preparations, and within the

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[3] Scholar in Cancer Research of the American Cancer Society, Inc.

limits of the applicability of the physical criteria, it could be concluded that the virus had been obtained in the purified state.

However, serious questions as to the validity of this conclusion were soon raised after two important observations. In the first place it was found that the plasmas of chicks with erythromyeloblastic leukosis were very active in the dephosphorylation of adenosinetriphosphate and that the enzyme was concentrated with the particles (*8*). Since no other virus was known to exhibit this behavior, the finding implied the possibility of a second particulate component not previously detected either in the plasma or in the concentrates. That this was not this case was demonstrated by the application of several criteria. It was found that adenosinetriphosphatase activity was proportional to and appeared in the circulating plasma simultaneously with the specific particles with onset of the disease (*7*). Moreover, the enzyme activity could not be separated from the infectious particles by either sedimentation or electrophoresis (*9, 10*). In more recent studies (*11*) it was shown that the enzyme was precipitated quantitatively with the virus particles in the presence of antiviral immune serum produced in the chicken. These observations served to associate the enzyme specifically with the virus particles and, as a consequence, to exclude the possibility of a particulate enzyme extraneous to the virus particles.

The second question that stimulated the investigations described in this report was one of still greater concern, for it was found that the supposedly pure concentrates contained material behaving immunologically like normal chick-tissue and Forssman antigens. This was particularly significant, since such materials of host origin might well be present as contaminants carried along with the virus through the purification procedures. Numerous variations of the methods of fractionation were employed in an effort to eliminate the antigens, but no lessening in their amount could be accomplished beyond the second cycle of sedimentation, at which time the plasma proteins had been removed. The question of a specific relationship between the virus particles and chick-tissue and Forssman antigens was raised when the results of continued experiments were considered. Accordingly, experiments were made to determine the measure of the relationship. The initial phase of these studies compared the physical properties of the virus particles with those of the hypothetical entity or entities bearing normal chick-tissue and Forssman antigenicity. This was done, as in the case of the analogous studies (*7, 9, 10*) of the enzyme, by investigating the relationship of the particles, as estimated by direct counts and by enzyme activity, to the chick-tissue and Forssman antigens observed on (1) ultracentrifugal fractionation of plasmas containing the virus; (2) comparative sedimentation of the various attributes in virus concentrates; and (3) electrophoresis of virus concentrates. In these experiments the sedimentation and electrophoretic properties of the antigens were indistinguishable from those of the virus particles. The final phase of the work was concerned with a quantitative serologic analysis of the antigenic constitution of the

virus *per se* based on the characteristic infectious properties of the agent. The results have indicated that both chick-tissue and Forssman antigens appear to be constituent parts of the virus particle and that both participate specifically in the immunologic phenomena detected and measured by virus neutralization, precipitation, and complement fixation. As a consequence, the findings are of significance not only in their bearing on the nature of the virus of erythromyeloblastic leukosis, but they provide, in addition, significant evidence of the homogeneity of the particulate material in the purified virus preparations. The results of the studies on the physical, chemical, and serologic relationships of the antigens to the virus particles are the subject of the present paper.

Materials and Methods

The Disease and the Virus

The condition under investigation was the BAI strain A of erythromyeloblastic leukosis derived originally from a case of neurolymphomatosis by Hall, Bean, and Pollard (*12*). The various manifestations of the disease have been described by these authors and by Johnson (*13*). In the experience of the latter investigator, transfer of the disease, *principally by whole-blood inoculums*, resulted in the occurrence of erythroleukosis, granuloblastosis (myeloblastosis), and various forms of lymphomatosis. Burmester (*14*) has studied the disease in this laboratory and has found no evidence of pathology other than that ascribable to myeloblastosis. The disease observed has thus appeared, under the conditions of rapid transfer by virus alone in very young chicks, to consist solely of myeloblastosis, and, because of the nature and time sequences of the experiments, lymphomatosis has not been a recognizable interfering factor. The work in this laboratory has been confined to material derived from a single chicken brought to Duke by Dr. E. P. Johnson, as previously noted (*4*), and, in the examination of approximately 600,000 blood smears from about 60,000 chicks, there has been no indication of erythroblastosis or of change in the character of the disease at any period in the course of study. In the intervening years, the disease has been passed more than 140 times by inoculums principally of filtered plasma, but occasionally, in the earlier work (*14*), with whole blood or washed primitive cells. Since 1952, transfer has been accomplished only with filtered plasma of high virus content obtained from chicks about 15 to 80 days of age, such as those described in tables 1 and 2 of reference 7. In most instances the passage virus was derived from the donor at the height of the disease within a few days of onset.

Virus.—The virus for the present studies consisted of the agent in the plasma of young chicks with the disease or in concentrates of the specific particles obtained by ultracentrifugation of the plasma. The donor chicks, which had received filtered plasma in various routine titration experiments, were selected for high plasma content of virus by means of the microscreening test (*15*, *16*) for adenosinetriphosphatase activity. Blood was obtained principally by heart puncture but occasionally by section of the

sagittal sinus. The blood was collected in chilled tubes containing a solution of crystalline heparin [heparin-sodium (100 units per mg.), Nutritional Biochemicals Corporation, Cleveland, Ohio] in a concentration of 50 mg. per ml. in Ca-free Ringer's solution, and 0.1 ml. of this solution was used for each 10 ml. of blood. The cells were removed by centrifugation in the routine way, and the plasma was filtered with celite and then passed through Selas filters. It should be emphasized that the virus was *always derived from plasma which had been filtered in this way.* Because of the small size of the birds, the volumes of plasma were usually not more than 1 to 3 or 5 ml. from each donor.

Titration of virus infectivity.—This was carried out as already described (*17*). The test hosts were 3-day-old White Leghorns of the highly inbred Line 15 (*18–20*). They were obtained both from a laying flock of this line of chickens in the laboratory at Duke and from eggs shipped from the Regional Poultry Research Laboratory, East Lansing, Michigan. All the eggs were incubated in this laboratory. The inoculum was invariably 0.1 ml. given intravenously in the wing vein. Potency ratios of various materials in the different experiments were calculated on the basis of the latent-period procedure (*17*).

Physical and Chemical Procedures

Concentration of the virus.—Since the present report is concerned with various aspects of the properties of virus concentrates, it would be well to describe the process of concentration for later discussion of certain of the factors influencing the outcome. The virus is routinely obtained by the centrifugal fractionation of plasma from diseased chicks, and the amount of the agent in this fluid governs, to a large extent, the character of the concentrates with respect to particle homogeneity.

The content of virus particles in the plasmas of chicks in the advanced stages of erythromyeloblastic leukosis, measured by infectivity (*10, 21*), particle count (*7*), or enzyme activity (*7, 16*) varies over a very broad range. The present technique for counting particles with the electron microscope is essentially inapplicable (because of high plasma-protein concentration and relatively large amounts of extraneous particles, as illustrated in figure 2 of reference 7) to plasmas of particle concentrations much below 10^9 per ml. Consequently, information about particle content below this number cannot be readily obtained by this means. It is known (*7*), however, that the variation extends from this level to about 10^{12} particles per ml., a range of approximately 1,000-fold. Unfortunately, the sensitivity of the determinations of the dephosphorylating-enzyme activity is likewise limited to about the same range (*7*). Estimation of enzyme activity can be made rapidly and simply in large numbers of birds, and since this property is a measure of particle number or mass, the best idea of the distribution of variation has been obtained in this way (*16*). The number of chicks with high virus content in the plasma is small, not more than 5 to 10 percent of the plasmas containing enough of it to justify application of purification procedures. It can be estimated by calculations

from particle count, average diameter—140 mμ (*22*)—and density of the hydrated particles—1.059 (*22*)—that the hydrated mass of virus per 10^{12} particles is about 1.5 mg. This would represent approximately 0.3 mg. of dry virus. Electron micrographs of plasmas containing 10^{11} to 10^{12} particles per ml. (see fig. 4) give the appearance (*7*) of great homogeneity of particle kind, and only such material has been employed for fractionation.

Initial sedimentation of the virus was effected by spinning 30 to 120 ml. of filtered plasma in 15 ml. celluloid tubes at 20,000 × g for 45 minutes. The pellets were resuspended in Ca-free Ringer's solution in appropriate volumes, usually about 1.0 ml. per 15 ml. of plasma. Low-speed centrifugation of the pellet suspension was carried out in the angle head of an International centrifuge for 10 minutes at 3,000 × g. After sampling, if desired, the pellet suspension was diluted to levels of about 4× concentration (based on the original volume of plasma), and the virus was sedimented for the second time at 20,000 × g for 30 minutes. The second-cycle pellets were taken up as before, spun at 3,000 × g for 10 minutes, diluted, and again sedimented at 20,000 × g for 30 minutes. These cycles of alternate high- and low-speed spinning were repeated 3 to 5 times. The final pellets were resuspended, spun at low speed, and diluted to appropriate volume.

Sedimentation of the virus from filtered plasmas in the first run yields clear pellets containing practically no opaque material. These are readily redispersible in Ca-free Ringer's solution with little or, occasionally, no evidence in electron micrographs of particle aggregation (fig. 1), and very little material extraneous to the characteristic particles can be seen. In many instances, the virus sedimented for the second time showed greater aggregation (fig. 2), and because of this accurate counting is not often feasible. Aggregation is increased still further (fig. 3) by a third sedimentation, and in electron micrographs the particles have lost some definition of form. Because of this behavior, nearly all the studies involving particle counts have been made, as will be indicated, with material sedimented a single time. This has introduced the complication, in the immunologic studies, of the presence of small amounts of plasma proteins as well as traces of some particles differing from the virus not eliminated in the single cycle.

It has been observed that aggregation can be avoided to a large extent by employing a solution containing 0.12 *M* NaCl and 0.17 *M* phosphate buffer of *p*H 7.0 as the dispersion medium in the fractionation process, instead of Ca-free Ringer's solution. This has been employed in special studies in which it was essential to count the particles in concentrates sedimented several times. The fractionation procedure was carried out in the sequence already described, with substitution of the phosphate-saline for Ca-free Ringer's solution as the only difference.

Comparative sedimentation.—It was desirable in one group of experiments to compare directly the rate of sedimentation of the virus particles with the corresponding rates of the chick-tissue and Forssman antigens. These studies were carried out by the same technique employed earlier (*9*, *10*)

in an analogous investigation of the sedimentation properties of the particles, the infectious unit, and the enzyme activity to dephosphorylate adenosinetriphosphate. The virus material for the present experiments consisted of concentrates obtained by a single sedimentation. Portions of the concentrate suspended in Ca-free Ringer's solution were placed in specially constructed, small cylindrical cells (*23*) which were then spun in the ultracentrifuge at 7,488 × g for varying periods of time. After the appropriate interval of centrifugation, the top layer of fluid was drawn off to the same level in all cases as determined by the length of the hypodermic needle employed for the purpose. By particle counts and titrations of chick-tissue and Forssman antigens in the withdrawn fluid, estimates were made of the rate of removal of the attributes from the fluid above the level of sampling. From these data, the respective rates of sedimentation could be calculated.

Electrophoresis.—Studies were made of the electrophoretic properties of the virus particles, the enzyme activity, and of the chick-tissue and Forssman antigens. The procedures were the same as those previously used in the studies of the particles, enzyme, and virus infectivity (*9, 10*)—deviating only in the use, for the present purpose, of virus concentrated in a single cycle of sedimentation. Sedimentation of the virus was essential, principally, in order to eliminate the large amount of plasma protein which would have interfered with the immunologic studies and, secondarily, to provide sufficient virus for photographic identification of the migrating virus boundary. The virus for each experiment was derived from pools of plasma from several chickens as described above. Dispersion of the pellets after the single cycle of ultracentrifugal concentration was effected in small volumes of the appropriate buffer and, after low-speed centrifugation, the concentrates, diluted to about 14 ml., were dialyzed overnight at 3° C. Phosphate buffer at *p*H 7.0 and veronal buffer at *p*H 8.5 were employed at 0.1 ionic strength. Photographs of the virus boundary were made at appropriate intervals during the experiment.

Migration in the divided, five-part cell was allowed to proceed until the visible boundary had passed just beyond the respective junctions of the upper and lower parts of the cell on the descending and ascending sides (see diagram of text-fig. 1 in reference 9). The contents of the five sections of the cell, upper cathode (UC), down cathode (DC), Cap, down anode (DA), and upper anode (UA) were sampled (*9, 10*), and determinations were made of particle content, chick-tissue antigenicity (as measured by complement fixation against antinormal chick-tissue immune serum from the rabbit), Forssman antigenicity, and activity to dephosphorylate adenosinetriphosphate. Calculations (*9, 10*) of the mobilities of the various components were made for both ascending and descending limbs of the cells, and the average value for the two limbs was obtained in the same way.

Enumeration of virus particles.—Counts were made of the virus particles by the technique of sedimentation on agar and electron micrography (*24*). The particles were fixed on the agar surface with osmic-acid vapor for at

least 5 minutes. It has been observed since the earlier reports (*6*, *7*) that the particles may not be removed quantitatively from the agar by a single stripping with the collodion film. As a consequence, it became the practice to obtain two films from each surface, and the values given in this paper represent the sum of the counts obtained with the two films. There has been no indication of need for a third stripping.

Enzyme activity.—Activity of the preparations to dephosphorylate adenosinetriphosphate was estimated by the measurement of liberated phosphorus with the method of Fiske and Subbarow (*25*). The reaction between adenosinetriphosphate and the appropriate volumes of the virus preparations was carried out in the presence of the composite saline solution (*26*) optimum for activity. The temperature of incubation of the mixtures was 30° C.

Serologic Materials and Procedures

Virus antigen.—The source of this antigen for the production of immune serums was that present either in plasma or in concentrates obtained by ultracentrifugation of plasma from diseased chicks, as will be described in the specific cases. Complement fixation, neutralization, and precipitation were carried out with virus concentrates and also with virus in plasma as indicated.

Chick-tissue antigen.—This antigen consisted of homogenates of mixtures of spleen and liver tissue from normal birds 1 to 2 months of age. These were predominantly New Hampshires from chosen, healthy, local flocks or White Leghorns of Line 15 raised in this laboratory. Usually the whole liver and whole spleen of the chicken were removed, washed, if necessary, with Ca-free Ringer's solution to remove gross blood, and ground in a chilled mortar with sand and Ca-free Ringer's solution. The tissue suspension was made to 10 percent with Ca-free Ringer's solution and spun, without prior standing, in the horizontal head of the International centrifuge at 600 $\times$ g for 10 minutes. The supernatant fluid, which was very turbid and viscous, was used as such for antigen.

Forssman antigen.—For the production of immune serum only, this was prepared from homogenates of guinea-pig kidney (*27*) in a manner like that employed for chick-tissue antigen.

Immune serums.—Antiviral immune serums were produced in rabbits, mice, guinea pigs, and chickens. Concentrated virus, obtained in 3 to 5 cycles of fractionation, was administered intraperitoneally in 0.5 ml. amounts (1.0 ml. equivalent to virus in 10 ml. plasma) in each of 4 doses at 3-day intervals in adult rabbits and guinea pigs; adult mice were given 0.25 ml. of the virus suspension in a single dose. Blood for serum was taken 7 to 14 days after the last injection; a second course of injections was made in some of the rabbits. Immune serum was produced in 3- to 4-month-old White Leghorns of Line 15. The initial injections consisted of concentrated virus treated with formalin in 0.1 percent concentration for 17 days. Each bird was given a series of 3 injections of 1.0 ml. of virus concentrate intravenously; after 8 weeks, a series of 3 more injections was made intravenously, each with 1.0 ml. of highly potent, untreated,

filtered *plasma* from diseased chicks. All serums were stored in 1.0 ml. volumes frozen at −15° C., except the mouse serum, which was kept in 5λ volumes sealed in melting-point tubes under the same conditions. No preservative was added to any of the serums.

Complement fixation.—The preliminary studies with complement fixation were made with the micro procedure previously described (*28*). In later studies it has been found more convenient to increase the final volumes of the mixtures to make possible estimates of hemolysis with the colorimeter. For the titration of antigen, serial twofold dilutions of virus concentrates, normal chick serum (or plasma), or extracts of normal chick tissue, as described above, were prepared in 10λ volumes and placed in tubes 5 mm. (ID) × 100 mm. To each there were added 20λ volumes of mixtures containing 3λ of immune serum and sufficient guinea-pig complement to lyse 90 percent of the test cells. After incubation of the mixtures overnight in an ice bath in the refrigerator, 0.8 ml. of a suspension containing 1.2×10^8 sensitized sheep cells per ml. was added to each tube. Final incubation was carried out at 37° C. for 30 minutes. The tubes were spun to sediment the intact red cells, and the supernatant fluids, taken off with fine-tipped pipettes, were diluted to 2.0 ml. volumes. Hemolysis was estimated with the Beckman Jr. spectrophotometer. Titration of the complement-fixing capacity of the immune serum was carried out in the same way employing optimal amounts of antigen and twofold dilutions of the serums. All immune serums were heated at 56° C. for 30 minutes immediately before use; *in all instances, except those involving titration of Forssman antigen, the serums were absorbed with sheep red blood cells for the removal of Forssman antibody.* For this purpose, 0.3 ml. of washed, packed (by centrifugation for 10 minutes at 1,500 × g) sheep red blood cells were mixed with 1.0 ml. of the immune serum and allowed to incubate for 1 hour at 37° C. The cells were removed by centrifugation. The process, employing the same volumes of red cells, was repeated 4 times in every study. Agglutination of the red cells was usually observed in the first and second treatments, but none was seen in the final mixtures. By this manner, the Forssman titer could be reduced, for example, from 256 to less than 2 in 3 steps of absorption.

Neutralization.—Virus for these tests was that present in pools of whole filtered plasma or, occasionally, in preparations of virus concentrated by a single sedimentation. The antiserums from rabbits or chickens were heated at 56° C. for 30 minutes, and the serums from rabbits were absorbed with sheep red cells as in the case of those employed for complement fixation. The details of the preparation of serum-virus mixtures are given with the individual experiments. For test of residual virus infectivity, the appropriate mixtures were injected in 0.1 ml. volumes intravenously in 3-day-old chicks. In the earlier experiments the chickens were New Hampshires, but all the studies since September 1, 1952, were made with White Leghorns of Line 15. The results observed in these experiments with White Leghorns were analyzed by the latent-period relation (*17*), yielding data of relatively high accuracy.

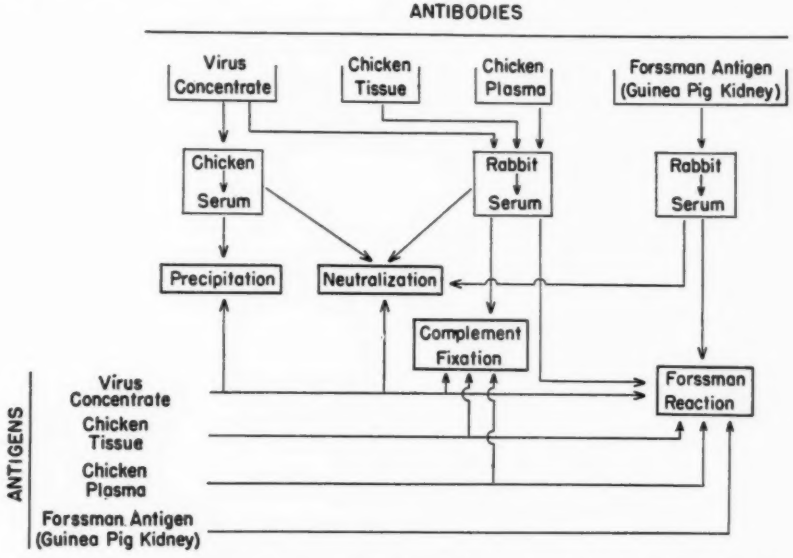
Precipitation.—The precipitin reaction was observed only with the antiviral immune serums from the chicken. Reaction mixtures were prepared in volumes of 0.1 ml. of virus concentrates, 0.2 ml. of 15 percent NaCl solution (*29*), and 0.1 ml. chicken serum or dilutions of it. Incubation was for 2 hours at 37° C., followed by standing in the refrigerator overnight. Further treatment for special studies is described in the appropriate place.

Experiments and Results

The experiments are arranged in three groups which are in accord both with the nature of the studies and with the chronological development of the problem. In the first section are the findings identifying qualitatively the antigens present in virus concentrates. The physical properties of the chick-tissue and Forssman antigens with respect to their sedimentation and electrophoretic behavior are described in the second group. The third section is devoted to the analysis of the antigenic constitution of the virus based on specific neutralization and precipitation of the agent. Attention is directed to text-figure 1, which summarizes diagrammatically the essence of the results of the immunologic investigations.

1. Antigenicity of the Virus Concentrates

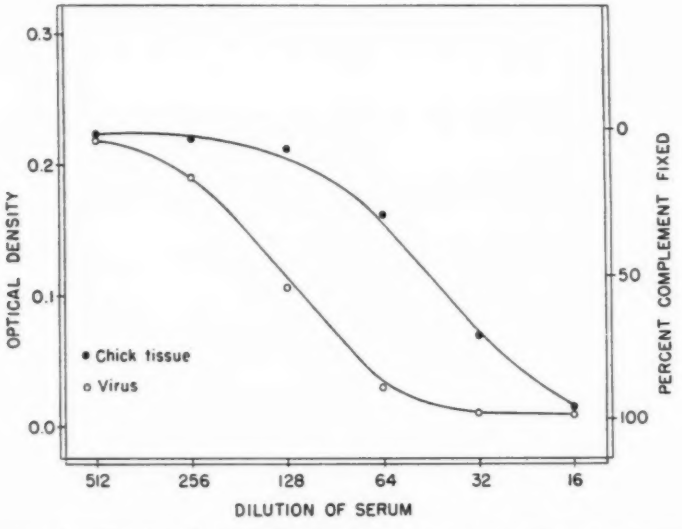
The preliminary studies on the immunologic properties of concentrates of the virus were made initially with two principal objectives: (1) to



TEXT-FIGURE 1.—Diagram of the reactions and cross reactions between virus concentrates, the chicken antigen of chicken plasma (which reacts in the same manner as normal chicken serum), the chicken antigen of chicken tissue, and the Forssman antigen (guinea-pig kidney) with the respective serums induced in the chicken and in the rabbit.

obtain immune serums of possible usefulness in the estimation of virus amount by complement fixation, precipitation, or other reactions; (2) to detect those nonviral antigens which might be present in purified virus preparations. It was to be expected that host antigens, particularly plasma proteins and, possibly, host tissue particles, would accompany the virus particles through a portion of the purification process. The plan was to employ an immunologic test as a criterion of the removal of such antigens. For this purpose purified viral concentrates procured from the plasma of diseased birds by 3 to 5 cycles of centrifugal fractionation were injected into rabbits as already described. Immune serums were induced in rabbits also by the administration of normal chicken serum and of homogenates of normal chick tissue. All these rabbit serums gave the complement-fixation reaction, but no evidence was seen of precipitation of any antigen under the conditions of the experiments. The studies of complement fixation and of the Forssman reaction with the rabbit immune serums are illustrated by the findings in the following experiments.

a) Complement-fixing antibodies induced in the rabbit by virus concentrates.—In text-figure 2 there are illustrated the results of the complement-fixation reaction between virus concentrate and antiviral immune serum. The serum had been heated at 56° C. for 30 minutes and Forssman antibody removed by absorption with sheep red blood cells. There was no evidence of nonspecific inhibition of the reaction in the range of antigen and antibody quantities employed for study. It is seen that the reaction between complement and concentrate was sharp, varying from complete to negative fixation through a narrow range of serum dilution.



TEXT-FIGURE 2.—Fixation of complement by virus concentrate and by homogenate of normal chick tissue in mixtures containing antiviral immune serum induced in the rabbit with virus concentrate.

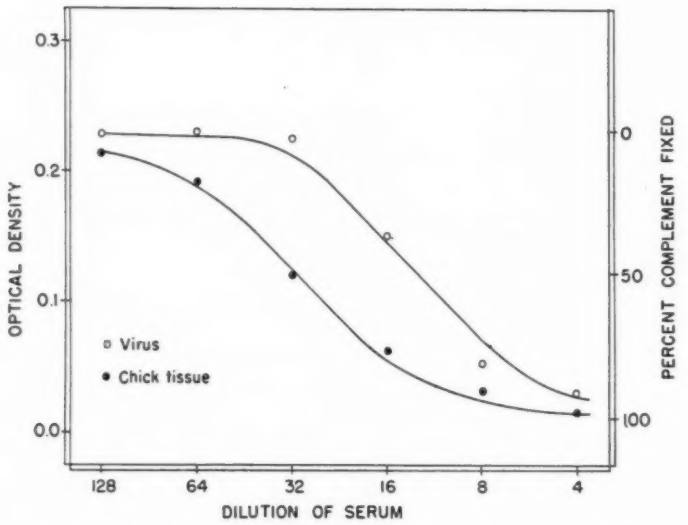
Further tests showed that the antiviral immune serum contained antibodies giving a like reaction with normal chicken tissue. The character of this reaction is described in the second curve of text-figure 2. It is evident that the pattern obtained with chick tissue was practically identical with that resulting with the virus.

The pattern of reaction in text-figure 2 was that observed uniformly to result in the studies on complement fixation with viral concentrates and with homogenates of normal chick tissues. A somewhat different pattern occurred, as will be seen, when normal chick serum or plasma was employed as antigen. In studies such as those of text-figure 2, it was found that while the total relation of fixation to serum dilution was sigmoid, there was an essentially linear relation in the region adjacent to the point of 50 percent fixation (or hemolysis). Furthermore, in the tests for comparing the relative titers of several preparations of the same material in the same experiment, the slopes of the lines were highly uniform. As a result, straight lines of constant slope could be fitted to the observed data, and the point of 50 percent hemolysis could be determined graphically to yield the value of the titer.

An analysis was made of the precision of estimates of complement-fixation titer under these conditions. The calculations showed that, with a 95 percent probability, the variation in end point to be expected in a single determination was approximately ± 0.09 log quantity of antigen or antibody, or ± 15 percent of the individual titer. It should be emphasized that this was the variation to be expected only in a comparison of a series of materials in the same experiment in which all of the reagents were identical. With repetition of titration on different days, greater variation was observed in absolute titer, but the proportionality of the titers remained the same.

b) Complement-fixing antibodies induced in the rabbit by homogenates of chick tissue.—Immune serums of this sort exhibited, as expected, the complement-fixation reaction in the presence of homogenates of normal chick tissue. Typical results of the phenomenon are shown in text-figure 3. Tests with viral concentrates against the same serum yield essentially identical patterns (text-fig. 3). Comparison of the curves of text-figure 3 with those of text-figure 2 reveals that the patterns of direct and cross reactions with the respective immune serums and antigens are closely similar. No significance can be placed on the differences in absolute titer which are largely dependent on the proportions of serum and antigen in the virus mixtures.

c) Complement-fixing antibodies induced in the rabbit by normal chick serum.—Normal chicken serum or plasma induced antibodies in the rabbit which could not be differentiated from those produced by homogenates of normal chick tissue. Such serums in the presence of viral concentrates or normal chick tissue yielded patterns of complement fixation like those observed with antiviral and antinormal chick-tissue serums (text-figs. 2 and 3). In contrast, however, normal chicken serum or plasma, when used as antigen in the titrations with antiviral, antinormal

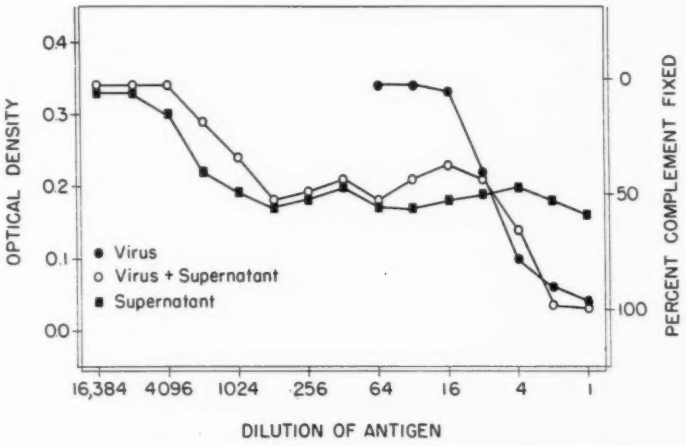


TEXT-FIGURE 3.—Fixation of complement by virus concentrate and by homogenate of normal chick tissue in mixtures containing antinormal chick-tissue immune serum induced in the rabbit with homogenate of normal chick tissue.

chick serum, and antinormal chick-tissue serums behaved in a different way. The difference is readily apparent from the curves of text-figure 4. In this experiment, a pool of plasma containing virus was obtained from several chicks and filtered as usual. The virus was concentrated in 1 cycle of high- and low-speed spinning. Some of the virus concentrate was returned to a portion of the supernatant plasma in the concentration originally present, and another aliquot of the concentrate was diluted to corresponding volume with Ca-free Ringer's solution. The complement-fixing capacity of each of the three preparations was now titrated with constant volumes of the immune serum.

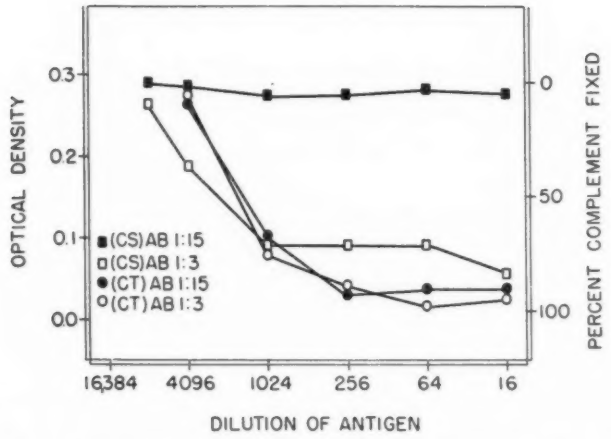
It is seen that the pattern of fixation by the virus concentrate is the same as the analogous patterns of text-figures 2 and 3. In contrast, despite an ultimate titer much higher than that of the virus, the fixation by the plasma from which the virus had been removed was never complete, even in the presence of large quantities of the antigen. This behavior of plasma from the diseased bird from which the virus had been removed was the same as that observed with normal chicken plasma or serum. Still another pattern was obtained with the plasma to which the virus had been returned. Here, the influence of the virus to yield sharp fixation is plainly evident in the mixtures containing the higher concentrations of the reconstituted virus-plasma preparation. In the region of high antigen concentration, the pattern of the plasma is overshadowed by the contribution of the virus. This effect of the latter declined rapidly in the region of the reaction by virus concentrate alone, and the pattern subsequently was that governed by the properties of the plasma antigen.

d) Influence of antigen-antibody proportions on the pattern of complement fixation.—It was observed that the pattern of complement fixation exhibited by plasma as antigen in reactions with immune serums, as illustrated in text-figure 4, was greatly influenced by the relative proportions of antigen and antibody. This was a phenomenon of major importance to the investigation. Since the virus occurred in chicken plasma, the protein content of which was physically and immunologically greatly in excess of the virus, the problem was always present of the contribution of plasma antigen to the complement-fixing behavior of the virus concentrates. Fortunately, interference of the plasma antigenicity could be greatly diminished or sometimes eliminated almost completely by proper adjustment of the antibody concentration. In the experiment of text-figure 5, chick-tissue homogenate was titrated as



TEXT-FIGURE 4.—Patterns of the complement-fixation reactions observed with concentrated virus, with plasma containing virus, and with plasma containing no virus in tests against antinormal chick-serum antibodies from the rabbit.

antigen against a series of two different dilutions, 1:3 and 1:15, of anti-chick-tissue immune rabbit serum. Similar titrations were made with normal chicken serum, which behaves in the same way as plasma in the complement-fixation reaction, as antigen against the same dilutions of immune serum. It is seen in text-figure 5 that with both dilutions of immune serum, the pattern of fixation with normal chick tissue was clear-cut and like the patterns shown in text-figures 2 and 3. In contrast, fixation by normal chicken serum was not complete even with the higher concentration of immune serum. The significant observation was that almost no fixation by the normal chick serum occurred with the smaller quantity of immune serum. Thus it was easily practical to select concentrations of immune serum optimal for sharp, complete reactions with either virus or normal chick-tissue homogenates but with small or no effect in the presence of normal chick serum or plasma. This phe-

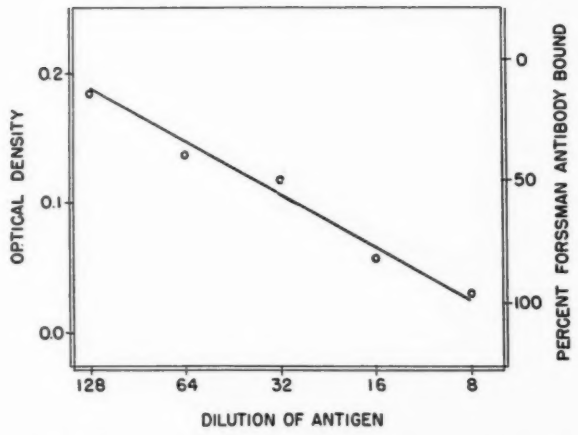


TEXT-FIGURE 5.—Influence of antigen-antibody proportions on the complement-fixing capacity of normal chick serum and of normal chick tissue in tests against antinormal chick-tissue immune serum from the rabbit.

nomenon made possible many serologic studies of the virus concentrates sedimented a single time.

e) *Forssman antigenicity.*—The tissues of the chicken exhibit the properties of Forssman antigen (*27*); in contrast, the rabbit is free of these heterophile substances. Studies of immune serums from the rabbit quickly revealed the presence of Forssman antibodies in animals immunized with virus concentrates or with normal chick-tissue homogenates. These antibodies, which lyse sheep red blood cells in the presence of complement, were found to react not only with normal chick tissue but actively with virus concentrates. The characteristics of the reaction are illustrated in text-figure 6. In this experiment, the Forssman antibody was that produced in the rabbit by immunization with homogenate of guinea-pig tissue (a source of Forssman antigen). An appropriate volume of this immune serum was mixed with varying dilutions of a virus concentrate sedimented a single time. After incubation of the mixture for 20 minutes at 37° C., sheep red blood cells were added. This was followed by further incubation for 30 minutes at 37° C., after which complement was introduced. The absorption of Forssman antibody by the viral concentrate is indicated by the curve of text-figure 6. The reverse reaction, namely, the reaction of Forssman antibodies produced in the rabbit with virus concentrate against guinea-pig-tissue antigen was likewise easily demonstrable. As a result of these phenomena, it was found essential in all experiments concerned with non-Forssman reactions to remove the Forssman antibodies by absorption with sheep red blood cells.

In contrast with virus concentrates and normal chick tissue, neither normal chick serum or plasma nor plasma from diseased birds, from which the virus had been removed, exhibited more than a trace of Forssman



TEXT-FIGURE 6.—Forssman reaction between virus concentrate and antibodies produced in the rabbit with homogenate of guinea-pig kidney.

antigenicity. Normal chicken serum tested as antigen against Forssman antibodies produced in the rabbit with guinea-pig tissue gave titers consistently less than 2. Analogous titrations with virus-containing plasmas from diseased birds gave titers in the range from 16 to 64.

f) Reaction with serums from mice and guinea pigs.—The tissues of both mice and guinea pigs, like those of the chicken, contain material of Forssman antigenicity (*27*), differing in this respect from the tissues of the rabbit. Because of these properties and of other factors, it seemed desirable in the early work to produce immune bodies in the mouse and the guinea pig. An important consideration was that of size. The virus was available only in small quantities, and, consequently, it was thought that relatively large doses of the agent per unit weight might produce more potent antiserums in the smaller animals. Furthermore, it was regarded as possible that the cross reactions between virus and anti-chick-tissue serum and between chick tissue and antivirus serums might be lessened both by species differences and by elimination of the Forssman reaction. Experiments showed that antiserums from mice and guinea pigs immunized with virus concentrate or normal chick tissue gave both direct and cross reactions with the respective antigens in the same pattern as that observed with immune serums from the rabbit. Such serums, however, contained no Forssman antibody. These results demonstrated no advantage of serum from immune mice and guinea pigs over immune serum from the rabbit, and all the subsequent work was concerned with reactions with rabbit serum and with serum from chickens.

Summation.—The foregoing experiments provided unequivocal evidence of the presence in viral concentrates of normal chick tissue and Forssman antigens. This was demonstrable in one direction by the capacity of the concentrates to produce in rabbits complement-fixing antibodies participating in cross reactions with normal chick antigen represented by either

tissue homogenates or normal chicken serum or plasma. Conversely, serums induced by the respective chicken antigens fixed complement with virus concentrates. Analogous reactions showed that virus concentrates could induce Forssman antibodies in the rabbit and, further, would behave as Forssman antigen in reactions with immune serum resulting in the rabbit from injection with homogenates of guinea-pig kidney. These findings are significant only in the identification of chick tissue and Forssman antigens in concentrates known to consist physically of the characteristic virus particles. Further experiments were necessary to determine the nature of the material responsible for antigenicity.

2. Sedimentation and Electrophoresis of the Virus Particles and of the Chick-Tissue and Forssman Antigens

It has already been stated that, in the procedures designed to concentrate and purify the virus, the final product has always contained virus infectivity, the specific virus particles, and, as well, material immunologically indistinguishable from normal chick-tissue and Forssman antigens. Inasmuch as the purification procedures have involved ultracentrifugal fractionation, the results implied the association of the chick-tissue and Forssman antigens either with a particulate material different from the virus particles or with the virus particles themselves. The purpose of the physical experiments has been to determine whether the hypothetical population of antigenic particles could be separated from the virus particles or, if not, to learn the degree of the physical relationship of the chick-tissue and Forssman antigens to the particles. The studies were of 3 kinds: (*a*) fractionation of plasmas from diseased birds by repeated cycles of high- followed by low-speed centrifugation; (*b*) comparison of the sedimentation rates of the particles with those of the chick-tissue and Forssman antigens; and (*c*) comparison of the rates of migration of the attributes on electrophoresis. Estimation of virus-particle content was effected by particle count or by both count and enzyme activity to dephosphorylate adenosinetriphosphate. Chick-tissue antigen was titrated by complement fixation in the presence of anti-chick-tissue immune serum from the rabbit, and Forssman antigen was measured with antiserum from rabbits immunized with homogenates of guinea-pig kidney.

a) *Ultracentrifugal fractionation.*—The distribution of virus particles—determined by direct count, of adenosinetriphosphatase activity, of chick-tissue antigen, and of Forssman antigen in the various fractions obtained by repeated sedimentation of the virus from plasma is illustrated in table 1. For this experiment a pool of 24 ml. of plasma was obtained from several diseased chicks and filtered in the usual way. Fractionation was carried through 3 cycles of sedimentation, employing the phosphate-saline solution for redispersion and dilution of the concentrates as described in the section on methods. In this study low-speed centrifugation was omitted after the first and second sedimentations. The suspension of the third concentrate, however, was spun in the angle centrifuge for 10 minutes at 3,000 $\times$ g. The supernatant fluid of this angle run, which contained

the virus, is designated in the table as the third concentrate. The corresponding sediment is indicated as the angle residue. From the results summarized in table 1, it is seen that the distributions of particles and adenosinetriphosphatase activity were closely similar in the different fractions, although variation occurred in the preparations of low particle content. The correlation was very good with the more concentrated pellet suspensions, which contained sufficient numbers of particles to permit more accurate counting. Estimation of chick-tissue antigen showed that this material was present uniformly in the pellet suspensions which were known to contain the particles. A loss of the chick-tissue antigen occurred between the first and second cycles of sedimentation but not thereafter. This phenomenon was related to the elimination of significant amounts of plasma protein (which behaved immunologically like chick-tissue antigen) in the second sedimentation. The Forssman antigen likewise followed the particulate material and, consequently, the chick-tissue antigen. Essentially neither antigen was observed in the supernatant fluids. The titer of the Forssman antigen in the concentrates remained constant throughout, differing in this respect from the chick-tissue antigen. Normal chicken plasma contains Forssman antigen only in very low titer, and the elimination of the small amounts of plasma in the second cycle would not be expected to contribute to significant change in the titer of the Forssman antigen between the first and second ultracentrifugal cycles.

b) Comparison of sedimentation rates.—In experiments like the one just described, the chick-tissue and Forssman antigens obviously followed the virus particles and were found only in those fractions containing the particles. Such results suggested a close similarity of the sedimentation properties of the materials exhibiting the various attributes. A better method for comparison of the sedimentation behavior of the particles and antigens consists in centrifugation of appropriate preparations for different intervals and estimating the rate of removal of the different properties

TABLE 1.—*The distribution of virus particles, adenosinetriphosphatase activity, chick-tissue antigen, and Forssman antigen in fractions obtained by 3 cycles of repeated ultracentrifugal sedimentations of the virus**

Fractions	Virus particles (*percent*)	ATPase activity (*percent*)	Chick-tissue antigen (*titer*)	Forssman antigen (*titer*)
Plasma	100	100	—	—
1st Concentrate	74	94	63	56
1st Supernatant	7	9	—	—
2nd Concentrate	97	98	28	60
2nd Supernatant	1	9	<2	<2
3rd Concentrate†	68	63	24	57
3rd Supernatant	0.1	13	<2	<2
Angle residue‡	4	12	—	—

*The volumes of all preparations were adjusted before test to equivalence with the initial volume of plasma. The calculations include the losses through sampling.

†Suspension of the third virus concentrate after angle centrifugation at 3,000 × g for 10 minutes.

‡Sediment resulting from angle centrifugation of the third concentrate.

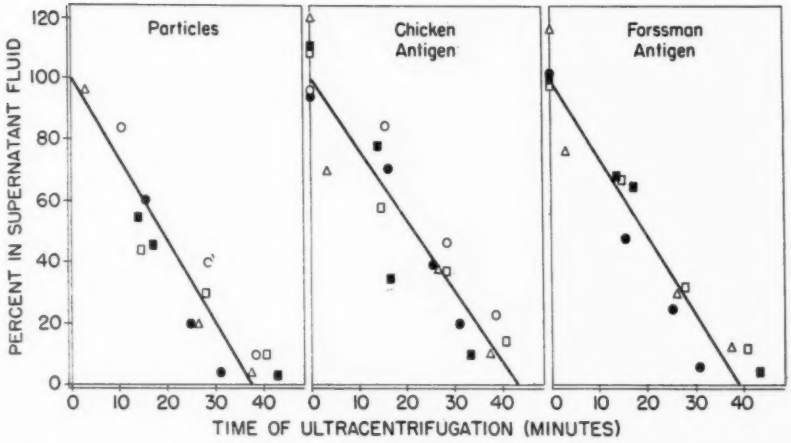
from a measured region of the centrifuge cell. A series of five studies was made with this procedure. The virus preparations consisted of concentrates obtained by one cycle of high- and low-speed sedimentation diluted to appropriate concentration with Ca-free Ringer's solution. Determinations of adenosinetriphosphatase activity were omitted because of the small amounts of material available. The particles were counted directly in the usual way, and the chick-tissue and Forssman antigens were titrated as before.

The results, table 2 and text-figure 7, showed that the particles and chick-tissue and Forssman antigens were sedimented at remarkably similar rates, especially within the individual experiments. It is notable, text-figure 7, that the chick-tissue antigen appeared to sediment somewhat more slowly than either the particles or the Forssman antigen. This was reflected in the respective sedimentation rates—607, 722, and 694—calculated from the data. The difference was probably real and due to residual amounts of plasma protein in the preparations. Plasma protein carried through into the concentrate, sedimented but once, would be expected to remain in constant concentration in all samples in a given experiment

TABLE 2.—*Distribution of particles, chick-tissue antigen, and Forssman antigen in virus preparations after various times of centrifugation at 7,488 × g*

Expt. No.	Time	Component content after centrifugation					
		Particles per ml.	Chicken antigen	Forssman antigen	Percent*		
	(*minutes*)	(× 10^{10})	(*titer*)	(*titer*)	Particles	Chicken antigen	Forssman antigen
3	0. 0	94	196	—	100	94	—
	15. 5	53	172	—	58	82	—
	26. 4	37	95	—	48	45	—
	38. 6	10	47	—	11	22	—
5	0. 0	298	500	127	100	109	102
	14. 4	131	250	83	43	54	66
	28. 4	92	170	40	30	37	32
	41. 1	33	69	15	10	15	12
6	0. 0	108	275	60	100	119	114
	3. 3	104	158	40	96	68	76
	26. 2	22	87	16	11	38	30
	37. 3	5	23	6	8	10	12
7	0. 0	181	186	105	100	95	102
	17. 2	110	138	50	61	71	48
	25. 9	36	76	26	20	39	25
	31. 5	18	39	6	10	20	6
8	0. 0	237	190	45	100	109	100
	13. 8	131	138	30	55	79	68
	17. 1	108	63	29	46	36	65
	43. 2	9	18	2	3	10	4. 5

*Calculations of the percent particles remaining after centrifugation were based on the number present in the concentrates before centrifugation as 100 percent. The values for chick-tissue and Forssman antigens were obtained by a different procedure. In this case the observed absolute titers were plotted against time of centrifugation, and a line was drawn through the points by the method of least squares. The value obtained graphically at the intercept of the line with zero time was used at the 100 percent level for converting all titers to percent.



TEXT-FIGURE 7.—Distribution of particles, chick-tissue antigen, and Forssman antigen in virus concentrates after various times of centrifugation at 7,488 × g. Each kind of symbol indicates the results obtained with the different attributes in the same experiment.

without relation to the time of centrifugation. Consequently, any complement-fixing effect contributed by the plasma would increase in relative importance as the virus was removed with increasing time of centrifugation. Considerable variation was seen in the absolute sedimentation rates of the materials from one experiment to another, a phenomenon related in all probability to differences in the viscosities of the various preparations. This did not lessen the significance of the results, since the principal point of interest was the interrelationships of the three attributes in the individual centrifuged samples.

c) Electrophoresis.—The techniques of these studies on the distribution of virus particles, adenosinetriphosphatase activity, chick-tissue antigenicity, and Forssman antigencity in electrophoretic fractions of virus preparations were the same as those employed in analogous studies on the relationships between virus infectivity, adenosinetriphosphatase activity, and particles previously described (*9, 10*). In the present case, the preparations were virus concentrated by a single sedimentation. This served the purpose of eliminating plasma proteins, which would have interfered with the serologic tests, and, in addition, of providing the virus in concentrations sufficient for the production of visible migrating boundaries.

The results of 4 experiments at *p*H 7.0 and 2 at *p*H 8.5 are given in table 3. A good impression of the findings is obtained by comparison of the distribution of the various attributes in the individual electrophoretic fractions within the same experiment. Variations were considerable in some instances from one experiment to another, but, as can be seen, these were entirely random in nature. On the whole, the distributions of the respective properties were remarkably uniform, considering the methods employed, particularly those involving the serologic titrations.

TABLE 3.—*Distribution of particles, enzymatic activity to dephosphorylate adenosinetriphosphate, chick-tissue antigen, and Forssman antigen in electrophoretic fractions of virus concentrates*

Expt. No.	Fraction	Particles per ml. ($\times 10^{10}$)	Enzyme* activity (γP)	Chicken antigen (*titer*)	Forssman antigen (*titer*)	Percent†				Visible boundary
						Particles	Enzyme	Chicken antigen	Forssman antigen	
1 pH 7.0	UC	7.1	11.5	7.8	29	15	11	26	17	
	DC	26.7	85.5	19.5	100	57	84	66	60	
	Cap	45.1	104.3	27.8	138	96	102	94	82	
	DA	50.6	100.0	22.2	166	108	98	75	99	
	UA	11.4	4.8	11.5	69	24	5	39	42	
			Mobility ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1})			−3.67	−2.93	−3.13	−3.71	−3.68
3 pH 7.0	UC	0.04	15.8	6.4	20.9	0.03	3	7.7	4.4	
	DC	95	350.8	62.9	309	77	70	75	65	
	Cap	121	517.4	72.5	549	98	104	87	116	
	DA	124	462.0	84.7	457	100	92	101	96	
	UA	30	154.1	24.0	89.3	25	31	29	19	
			Mobility ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1})			−4.03	−4.07	−4.02	−3.99	−4.07
4 pH 7.0	UC	3.1	43.5	3.5	7.4	3.1	8.9	10	1.9	
	DC	77	385.0	21.9	362	78	79	63	91.3	
	Cap	92	457.0	33.1	397	93	94	95	100	
	DA	101	431.0	31.0	362	102	88	89	91.3	
	UA	24	146.0	15.1	61	24	30	43	15	
			Mobility ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1})			−4.26	−4.01	−4.51	−3.72	−4.20
5 pH 7.0	UC	3.7	0.7	4.1	13.5	4	0.3	11	6	
	DC	64	206.9	31.7	246	70	88	85	110	
	Cap	92	212.5	35.5	200	101	90	95	90	
	DA	94	226.4	29.5	159	103	96	79	71	
	UA	21	58.1	11.2	51.3	23	25	30	23	
			Mobility ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1})			−5.16	−4.78	−4.37	−3.65	−4.70

	UC	1.9	5.4	7	6	5	6	8	3	
	DC	35	81.0	71	208	81	87	79	87	
10	Cap	42	102.2	87	238	97	110	97	100	
*p*H 8.5	DA	43	87.8	85	196	99	94	94	81	
	UA	7.9	2.3	20	69	18	3	22	29	
			Mobility ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1})			−3.77	−3.31	−4.12	−3.59	−3.70
	UC	1.0	1.4	4	5	2	3	9	2	
	DC	55	44.4	32	234	82	89	69	73	
13	Cap	66	48.6	43	347	99	98	93	108	
*p*H 8.5	DA	66	46.6	48	331	99	94	104	103	
	UA	12.3	7.9	12	45	18	16	25	14	
			Mobility ($\times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1})			−3.61	−3.36	−3.89	−3.71	−3.44

*γ Phosphorus liberated by 1 ml. of fraction in 1 minute.

†The percent value for a given cell section was calculated as the ratio of that value to the value of the control. The control was taken as one third of the total of the values for all five cell sections.

In each of these experiments, there was produced a visible boundary corresponding to the migrating particles. Occasionally there was also evidence of the characteristic electrophoretic pattern of traces of the plasma proteins. This was soon lost with the separation of the proteins, leaving only the diagram related to the virus particles. The characteristics of the consistently single, sharp boundaries are illustrated by the diagram of figure 8. This peak, occurring on the ascending side, was photographed at the end of experiment 3 (table 3), just after the boundary had passed the junction of the lower (DA) and upper (UA) anode sections (*9*) of the Tiselius cell. Such small boundaries were seen in all of the experiments. Efforts to obtain larger peaks were unavailing, in part because of excessive light scattering but more so because of the tendency of the preparations to gel. Whether this was related to residual plasma fibrinogen or to intrinsic properties of the virus in high concentration was not determined.

The mobilities of the particles, enzyme, chick-tissue antigen, and Forssman antigen were calculated (see reference 9 for procedure) from the data of table 3, and the resulting values are summarized in table 4. Whereas variation and differences in the absolute mobilities occurred in the different experiments, the values were quite similar in the individual studies. The correlation was very close in the average values. The similarity of the mobilities calculated for the test attributes to the mobility of the visible migrating boundary in each instance is clearly evident.

Summation.—The results of the sedimentation studies demonstrate quite clearly that the entity or entities bearing the chick-tissue and Forssman antigens are particulate (as contrasted with hypothetical materials of low molecular weight). Moreover, the particles constituted a specific population separable by centrifugation from the plasma proteins, which behave strongly, though in a characteristic way (see text-fig. 4), like chick-tissue antigen but display little Forssman antigenicity. Furthermore, these particles exhibit sedimentation properties essentially identical with those of the virus particles. An analogous similarity was observed

TABLE 4.—*Summary of the mobilities* ($\times 10^{-5}$ *cm.*^{2} *sec.*$^{-1}$ *volt*$^{-1}$) *of the virus particles, adenosinetriphosphatase, chick-tissue antigen, Forssman antigen, and of the visible boundary in the electrophoretic experiments at* p*H 7.0 and 8.5*

Experiment	Particles	Enzyme	Chicken antigen	Forssman antigen	Visible boundary
1. *p*H 7.0	−3. 67	−2. 93	−3. 13	−3. 71	−3. 68
3. "	−4. 03	−4. 07	−4. 02	−3. 99	−4. 07
4. "	−4. 26	−4. 04	−4. 51	−3. 72	−4. 20
5. "	−5. 16	−4. 78	−4. 37	−3. 65	−4. 70
Averages	−4. 28	−3. 95	−4. 01	−3. 77	−4. 16
10. *p*H 8.5	−3. 77	−3. 31	−4. 12	−3. 59	−3. 70
13. "	−3. 61	−3. 36	−3. 89	−3. 71	−3. 44
Averages	−3. 69	−3. 33	−3. 65	−4. 00	−3. 57

in the experiments by electrophoresis, which failed to reveal significant differences between the mobilities of the two antigens and those of the virus particles. There could be no question that the visible boundary was produced by the virus particles, and since the boundary, though small, was consistently single and sharp, the particles with the properties of chick-tissue and Forssman antigens must have been included with the population of virus particles. It could be estimated that in figure 8, a secondary component of sharpness which was similar to the material yielding the boundary shown and one fifth the concentration of that material would have been detectable in the diagram. No evidence was seen of such a hypothetical component. From the physical findings it can be assumed, with complete assurance, that the chick-tissue and Forssman antigens are associated with particles possessing sedimentation and electrophoretic properties identical with those of the virus particles. This constitutes strong presumptive evidence identifying the antigenic particles with those of the virus and serves to focus attention on the possibility that the antigens may be adsorbed on or may be integral parts of the virus particles themselves. While the results provide the basis for such concept, they provide no information on the character of the association. This aspect of the problem, however, is subject to critical experimentation by biological methods, and it was this which led to the immunologic studies described in the following section.

3. *Antigenic Constitution of the Virus as Demonstrated by Specific Neutralization and Precipitation*

The problem for solution in the following experiments was concerned with the application of serologic criteria suitable for demonstration of the nature of a possible relationship between the materials of virus concentrates responsible for the respective antigenic properties. Any criterion of virus presence and amount must be related, ultimately, to the single property, infectivity, known to be characteristic of the agent. Although complement fixation and precipitation and even physiochemical behavior may be employed as suitable bases for detection and estimation, these must be related first to infectivity. As a corollary, specificity in serologic reactions may be demonstrable by immunologic phenomena involving virus neutralization. These obvious premises have been applied to the measurement of the relationships of the normal chick-tissue and Forssman antigens to the virus particles with which they have been found consistently to be associated in concentrates of the virus. The studies have involved the neutralization reaction with immune serums induced in the rabbit and with specific neutralization and precipitation with analogous serums from the chicken.

a) Virus neutralization with antiviral immune serum from the rabbit.— The activity of the serum from rabbits immunized with viral concentrates to fix complement with viral concentrates was illustrated in various instances already cited, as for example in text-figure 2. That at least a portion of the immunologic activity of such serums was directed toward specific reaction with the virus is indicated by the results of the neutraliza-

tion study of text-figure 8. This was a checkerboard experiment in which a series of four different amounts of virus were set up with successive twofold dilutions of antiserum. As usual the serum had been heated at 56° C. for 30 minutes and the Forssman antibody absorbed with sheep red blood cells. The virus was that present in a pool of filtered plasma obtained from several diseased birds. The pattern of the experiment and the resulting data are summarized in table 5.

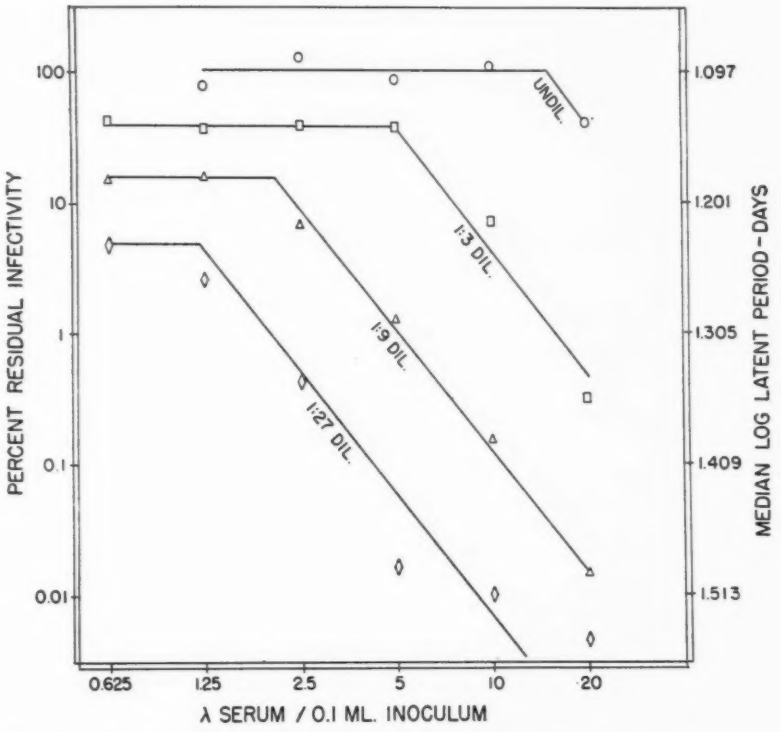
In text-figure 8, the individual points represent values of the mean log latent period derived from the respective individual dose-response relations in the manner described in another report (*17*). Control preparations consisting routinely of untreated virus were included to establish the starting level of infectivity. With this system there was available only one point to determine the level. A different method was finally adopted for locating the 100 percent level of infectivity, namely, the use of the value found with the untreated control together with those of all points obviously in the immediate range of highest infectivity. For

TABLE 5.—*Neutralization of virus in successive dilutions of filtered plasma with serum from a rabbit immunized with virus concentrate*

Serum	Virus plasma* (λ)	No. particles † ($\times 10^6$)	Immune serum* (λ)	Chicks inoculated‡	Chicks positive	Median log latent period (*days*)	Residual infectivity § (*percent*)
None	20	326	—	25	24	1. 098	98
Antiviral	20	326	20	24	24	1. 138	41
	"	"	10	26	24	1. 093	110
	"	"	5	24	24	1. 103	87
	"	"	2. 5	27	27	1. 087	126
	"	"	1. 25	25	25	1. 108	78
None	6. 7	108	—	24	23	1. 136	43
Antiviral	6. 7	108	20	25	16	1. 363	0. 3
	"	"	10	25	17	1. 215	7. 2
	"	"	5	28	24	1. 143	36
	"	"	2. 5	29	29	1. 140	39
	"	"	1. 25	29	26	1. 142	37
	"	"	0. 625	23	23	1. 136	42
None	2. 2	36	—	25	24	1. 166	16
Antiviral	2. 2	36	20	26	12	1. 495	0. 02
	"	"	10	28	18	1. 390	0. 2
	"	"	5	28	22	1. 294	1. 3
	"	"	2. 5	28	26	1. 219	6. 7
	"	"	1. 25	25	24	1. 180	16
	"	"	0. 625	24	20	1. 183	15
None	0. 74	12	—	25	25	1. 224	6. 0
Antiviral	0. 74	12	20	23	7	1. 548	0. 005
	"	"	10	22	8	1. 512	0. 01
	"	"	5	26	13	1. 485	0. 02
	"	"	2. 5	30	20	1. 343	0. 4
	"	"	1. 25	27	22	1. 262	2. 5
	"	"	0. 625	30	23	1. 235	4. 7

*Volume per 0.1 ml. inoculum.
† Number of virus particles per inoculum.
‡ Corrected number of chicks inoculated [see (*17*) for method of calculation].
§ Calculated on the basis of latent-period relations.

example, in table 5, there were employed the control value (1st line) and four values, 3rd to 6th lines, observed with mixtures of undiluted virus with the higher graded dilutions of serum. The average of these five (in this experiment) estimates served to establish the 100 percent level of infectivity. While there exists no well-established basis for such usage, it is obvious that the combination of all these values provides a much better estimate of the level than a single "control" value of precision no greater than that of any other individual point, as can be seen by inspec-



TEXT-FIGURE 8.—Neutralization of virus in filtered plasma and in three successive threefold dilutions of the plasma by antiviral immune serum from the rabbit (see table 5).

tion of the relations in text-figure 8. In the ordinate at the left, there is indicated the residual infectivity of the various preparations in terms of percent plotted on log scale. These percentage values were derived from the previously determined standard relations (*17*) between median log latent period and virus potency. This is such that a difference of 0.104 units of log latent period signify a tenfold difference in virus potency, which was fully substantiated in the present experiments in analyses of data obtained with different dilutions of the test-virus preparations. An illustration is seen in the experiment of text-figure 8 in which

the studies were made with undiluted plasma and three successive threefold dilutions of the plasma.

Some of the values given in the table were not plotted in the graph. These represented noncontributory data obtained with the control preparation and with the highest dilutions of serum which produced no neutralizing effect. The omitted data are employed, however, in the estimate of the percent level of infectivity. This practice was followed in the preparation of all of the graphs concerned with neutralization.

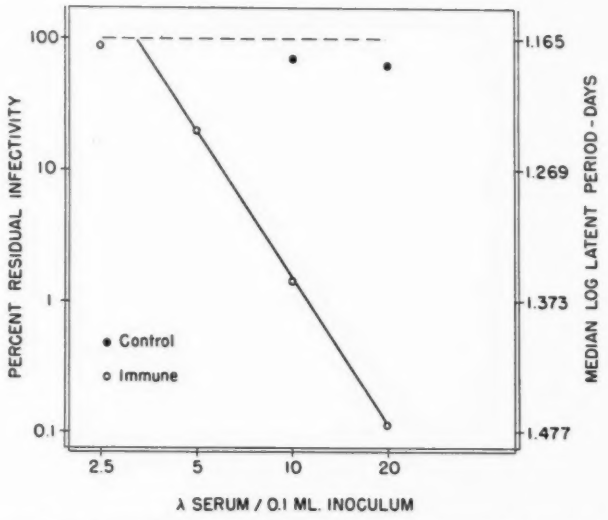
The activity of the antiviral rabbit serum to neutralize the agent is clearly shown. The data likewise reveal evidence of the precision of the method for estimation of infectivity. This was especially notable with the control preparations and with the serum-virus mixtures in which no neutralization occurred. More variation was seen with the mixtures of lowest infectivity because of the smaller numbers of affected chicks available for analysis. It is apparent that the basic pattern of neutralization was the same with all amounts of virus. In the region of neutralization, the relation of residual virus infectivity (or, conversely, the amount of virus neutralized) expressed on log scale of percent was essentially linear with serum dilution plotted on log scale. Furthermore, the lines in this region exhibited, for practical purposes, a constant slope.

A principle of critical importance in the neutralization studies is apparent in text-figure 8, namely, the dependence of the sensitivity of the data on the proportions of serum and virus. With the largest quantity of virus, only the largest volume of serum caused change, and this might have been missed if no other proportions had been employed. More definite results were obtained with a threefold dilution of virus, and data optimum for interpretation were observed with the ninefold dilution. With the 27-fold dilution of virus, the chicken response was too small for accurate analysis, though the neutralizing activity of the serum was well demonstrated. It was fortunate in these studies, which were tedious and costly, that virus concentration could be estimated and adjusted with considerable accuracy in advance by preliminary measurement of adenosinetriphosphatase activity.

As noted in table 5 and in the subsequent tables describing the neutralization experiments, there are given the numbers of virus particles in the various preparations. With these data, calculations may be made in all cases of the number of particles *apparently* neutralized. In the ninefold dilution of the plasma in table 5, for example, there were present 36×10^8, 3.6 billion, particles per inoculum. The ratio of residual virus, 0.02 percent, to the amount at the beginning, 16 percent (based on the 1:9 dilution of the original plasma preparation), is 1:800. In consequence, it is seen that the number of particles neutralized was 35.96×10^8, 3.596 billion, leaving the number active, in effect, only 0.045×10^8 or 4.5 million. These values, which emphatically illustrate the high potency of the neutralizing antibodies of the rabbit immune serum, should be regarded only as relative approximations. There was no way of knowing the proportion of active to, possibly, inactive particles in the untreated

preparations. The design of the experiments was not intended to produce complete neutralization but to limit the investigation to the range in which the residual activity affected a sufficient number of chicks for accurate analyses.

b) Virus neutralization with antinormal chick-tissue immune serum from the rabbit.—It will be recalled that the serum from the rabbit immunized with homogenate of normal chicken tissue possessed the capacity to fix complement with virus concentrates. Experiments were made to determine whether such serums might act likewise to neutralize virus infectivity. The make-up of the study and the data are given in table 6, and the results are shown graphically in text-figure 9. The serum had been heated at 56° C. for 30 minutes and absorbed with sheep red blood cells.



TEXT-FIGURE 9.—Neutralization of virus in filtered plasma by antinormal chick-tissue immune serum from the rabbit (see table 6).

In this experiment there were included mixtures containing the serum of the normal rabbit before immunization with chick tissue. With the normal serum there was little, if any, depression of the level of infectiousness. In contrast, the anti-chick-tissue immune serum was strongly neutralizing, reducing the level of infectivity by approximately 99.9 percent. It is notable that the pattern of neutralization by anti-chick-tissue immune serum is identical with that by antiviral immune serum, as seen by comparison with text-figure 8. Another example will be seen in text-figure 10 describing the next experiment.

The lack of neutralizing or inhibiting capacity of heated normal rabbit serum indicated in this experiment has been corroborated in numerous similar studies. Examination of many normal rabbit serums has not revealed an instance of significant activity in this respect.

TABLE 6.—*Neutralization of the virus in filtered plasma with the serum from a rabbit immunized with homogenate of normal chick tissue*

Serum	Virus plasma* (λ)	No. particles† ($\times 10^8$)	Immune serum* (λ)	Chicks inoculated‡	Chicks positive	Median log latent period (*days*)	Residual infectivity§ (*percent*)
None	10	150	—	25	23	1. 113	—
	2	30	—	25	21	1. 166	102
Normal	2	30	20	20	17	1. 187	62
	"	"	10	24	21	1. 176	78
Anti-chick-tissue	2	30	20	27	11	1. 469	0. 12
	"	"	10	24	14	1. 358	1. 4
	"	"	5	23	19	1. 233	22
	"	"	2. 5	27	24	1. 170	89
	"	"	1. 25	27	25	1. 168	93
	"	"	0. 625	21	19	1. 160	112

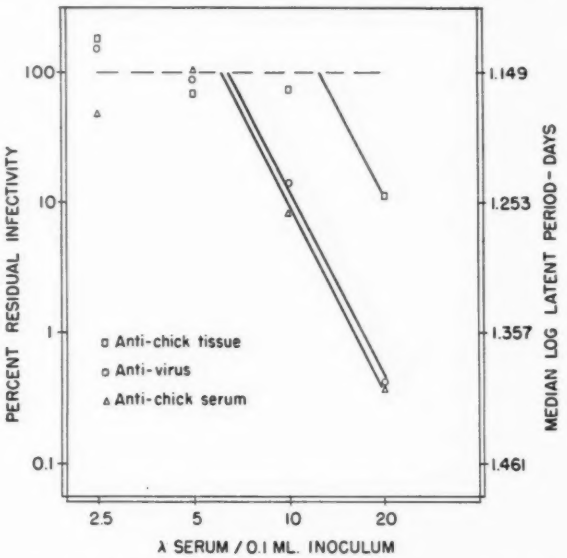
*Volume per 0.1 ml. inoculum.
†Number of virus particles per inoculum.
‡Corrected number of chicks inoculated [see (*17*) for method of calculation].
§Calculated on the basis of latent-period relations.

TABLE 7.—*Neutralization of the virus in filtered plasma with serums from rabbits immunized, respectively, with virus concentrate, with homogenate of normal chick tissue, and with normal chicken serum*

Serum	Virus plasma* (λ)	No. particles† ($\times 10^8$)	Immune serum* (λ)	Chicks inoculated‡	Chicks positive	Median log latent period (*days*)	Residual infectivity§ (*percent*)
None	4	24	—	29	28	1. 150	98
	0. 4	2. 4	—	28	25	1. 264	—
Antiviral	4	24	20	28	22	1. 396	0. 4
	"	"	10	22	19	1. 238	14
	"	"	5	22	20	1. 155	87
	"	"	2. 5	21	21	1. 131	151
	"	"	1. 25	20	19	1. 148	102
	"	"	0. 625	26	25	1. 142	118
	"	"	0. 312	24	23	1. 159	79
Anti-chick-tissue	4	24	20	23	20	1. 248	11
	"	"	10	22	19	1. 163	74
	"	"	5	24	23	1. 165	71
	"	"	2. 5	24	24	1. 120	190
	"	"	1. 25	22	20	1. 152	93
	"	"	0. 625	23	23	1. 124	174
	"	"	0. 312	25	25	1. 141	120
Antinormal chick	4	24	20	16	11	1. 398	0. 3
	"	"	10	21	19	1. 262	8. 1
	"	"	5	17	16	1. 148	102
	"	"	2. 5	27	23	1. 180	50
	"	"	1. 25	22	22	1. 155	87
	"	"	0. 625	21	19	1. 143	114
	"	"	0. 312	24	23	1. 173	59

*Volume per 0.1 ml. inoculum.
†Number of virus particles per inoculum.
‡Corrected number of chicks inoculated [see (*17*) for method of calculation].
§Calculated on the basis of latent-period relations.

c) Virus neutralization with serums from rabbits immunized with normal chick serum, with virus, and with normal chick tissue in the same experiment.—An experiment was made to determine the neutralizing effect of immune serum produced in the rabbit with normal chicken serum. For comparison, and for corroboration of other findings, similar tests were set up with antiviral and antinormal chick-tissue immune serums as indicated in table 7. The graphic representations of the data in text-figure 10 illustrate full substantiation of the findings in text-figures 8 and 9 and, in addition, establish clearly the pronounced neutralizing capacity of the antinormal chick-serum immune bodies. Both antinormal chick serum and antiviral immune serums neutralized more than 99 percent of the virus. It is clear in this experiment that the patterns of virus neutralization by the 3 different immune serums are identical.



TEXT-FIGURE 10.—Neutralization of virus in filtered plasma with immune serums from rabbits immunized with normal chick serum, with virus, and with normal chick tissue (see table 7).

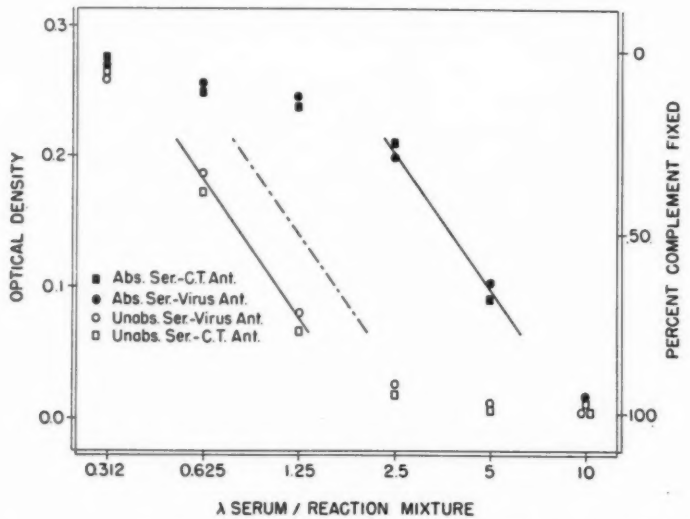
d) Absorption of complement-fixing and neutralizing antibodies from antiviral immune serums from the rabbit with normal chick tissue.—The results of absorption with normal chick tissue of immune serum produced in the rabbit by injections with virus concentrate are revealed in table 8 and text-figures 11 and 12. Mixtures of virus in filtered plasma were prepared with heated Forssman-absorbed immune serum as indicated in the table.

Absorption of antibodies with normal chick tissue was effected by the following procedure: A large volume of 10 percent suspension of homogenized chick tissue, described earlier in this paper, was pipetted into 50

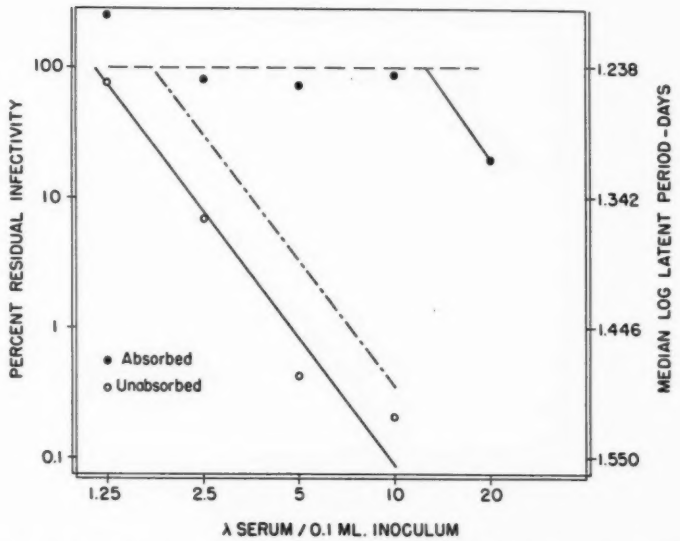
TABLE 8.—*Neutralization of the virus in filtered plasma with the serum from a rabbit immunized with virus concentrate and with the same immune serum absorbed with homogenized chick tissue*

Serum	Virus plasma* (λ)	No. particles† ($\times 10^8$)	Immune serum* (λ)	Chicks inoculated‡	Chicks positive	Median log latent period (*days*)	Residual infectivity§ (*percent*)
None	1	8.4	—	25	22	1.205	204
	0.1	0.84	—	22	16	1.335	—
Antiviral	1	8.4	10	24	5	1.518	0.2
	"	"	5	27	13	1.484	0.4
	"	"	2.5	23	16	1.359	6.9
	"	"	1.25	27	25	1.250	78
	"	"	0.625	27	21	1.276	42
	"	"	0.312	25	21	1.228	126
Antiviral (absorbed)	1	8.4	20	29	16	1.311	20
	"	"	10	22	20	1.243	89
	"	"	5	25	22	1.251	74
	"	"	2.5	28	24	1.247	81
	"	"	1.25	18	18	1.195	257
	"	"	0.625	22	19	1.232	115
	"	"	0.312	26	20	1.237	102
	"	"	0.156	27	20	1.232	115

*Volume per 0.1 ml. inoculum.
† Number of virus particles per inoculum.
‡ Corrected number of chicks inoculated [see (17) for method of calculation].
§ Calculated on the basis of latent-period relations.



TEXT-FIGURE 11.—Absorption of complement-fixing antibodies from antiviral immune rabbit serum by homogenate of normal chick tissue in tests against virus in filtered plasma. The results of parallel studies on the absorption of neutralizing antibodies in the same experiment are shown in text-figure 12. Maximal influence due to nonspecific absorption or dilution in the absorption process, indicated by the broken line, was derived from estimates with pneumococcus polysaccharide type 8 antibodies included in the mixtures.



TEXT-FIGURE 12.—Absorption of neutralizing antibodies from antiviral rabbit immune serum by homogenate of normal chick tissue (see table 8). The results of parallel studies on the absorption of complement-fixing antibodies in the same experiment are shown in text-figure 11. Maximal influence due to nonspecific absorption or dilution in the absorption process, indicated by the broken line, was derived from estimates with pneumococcus polysaccharide type 8 antibodies included in the mixtures.

ml. collodion centrifuge tubes. The material was spun at 3,000 × g in the angle head of the International centrifuge No. 2 in the cold for 30 minutes. After the supernatant fluid had been poured off, the sediment was made up to the initial volume with Ca-free Ringer's solution and thoroughly stirred. Sedimentation and resuspension were repeated 4 times. Following the 4th sedimentation, the supernatant fluid was poured off, the tube was well drained, and the wet, washed tissue residuum was weighed. To a portion of the immune serum, 4 ml. diluted to 20 ml. with Ca-free Ringer's solution, there were added 6 gm. of the packed sediment. The mixture was incubated for 2 hours at 37° C. and then left overnight in the refrigerator. The chick tissue was sedimented by spinning in a 50 ml. tube for 30 minutes at 3,000 × g in the angle head. After this procedure had been repeated 3 times, with fresh packed tissue in each instance, the absorbed serum was carefully pipetted from the sediment. The absorbed serum was spun once again at 3,000 × g for 30 minutes, removed by pipetting, and finally centrifuged at 20,000 × g for 1 hour. This yielded a small gelatinous pellet from which the absorbed immune serum was separated with fine-tipped pipettes.

In order to obtain an estimate of the nonspecific absorption or dilution of antibody by this process, pneumococcus polysaccharide type 8 antibody was added to the immune serum before absorption. Titration of the anti-

body by complement fixation against the specific antigen before and after absorption provided the data for calculation of the amount lost in the procedure.

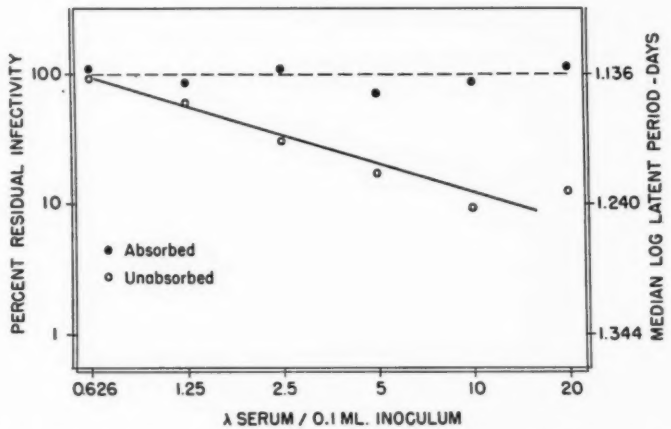
The data of the graph of text-figure 11 indicated a total reduction in the complement-fixing capacity of the antiviral immune serum of about 80 percent as the result of absorption. The extent of the concurrent diminution in neutralizing activity, text-figure 12, was 90 percent. Calculations from the respective titers of the pneumococcus antiserum in the absorbed and unabsorbed immune serum revealed a possible maximum loss of about 33 percent of the complement-fixing and neutralizing antibodies related to nonspecific absorption or dilution, as shown by the broken lines of text-figures 11 and 12. Consideration of the increase in the volume of immune serum during the absorption process indicated that the decline in activity was related more to dilution than to nonspecific absorption. Adjustment of the observed titers of complement fixation and neutralization gave values of 67 and 85 percent as estimates of specific absorption of the respective antibodies. It may be mentioned here that results such as these have received substantiation in numerous like experiments made during the past 2 years.

e) Virus neutralization with Forssman antibody induced in the rabbit with guinea-pig tissue.—The outcome of the preceding experiment indicated that Forssman antibody, like the antinormal chick-tissue immune serum,

TABLE 9.—*Neutralization of the virus in filtered plasma with Forssman antibody in the serum of a rabbit immunized with homogenate of guinea-pig kidney and with the same serum absorbed with sheep red blood cells*

Serum	Virus plasma* (λ)	No. particles† ($\times 10^8$)	Immune serum* (λ)	Chicks inoculated‡	Chicks positive	Median log latent period (*days*)	Residual infectivity§ (*percent*)
None	2	47	—	23	21	1. 128	120
	0. 2	4. 7	—	23	15	1. 236	—
Anti-Forssman	2	47	20	23	22	1. 230	13
	"	"	10	26	19	1. 242	10
	"	"	5	20	18	1. 215	17
	"	"	2. 5	23	21	1. 190	30
	"	"	1. 25	25	22	1. 159	60
	"	"	0. 625	17	15	1. 139	93
	"	"	0. 312	24	21	1. 151	72
	"	"	0. 156	24	22	1. 136	100
Anti-Forssman (absorbed with sheep red cells)	2	47	20	27	25	1. 130	115
	"	"	10	25	24	1. 142	87
	"	"	5	23	18	1. 152	71
	"	"	2. 5	20	17	1. 132	110
	"	"	1. 25	22	17	1. 143	85
	"	"	0. 625	24	22	1. 132	110
	"	"	0. 312	27	26	1. 127	123
	"	"	0. 156	23	22	1. 139	93

*Volume per 0.1 ml. inoculum.
†Number of virus particles per inoculum.
‡Corrected number of chicks inoculated [see (*17*) for method of calculation].
§Calculated on the basis of latent-period relations.



TEXT-FIGURE 13.—Neutralization of virus in filtered plasma by Forssman antibody in the serum of a rabbit immunized with homogenate of normal guinea-pig kidney, and the absorption of the neutralizing Forssman antibody with sheep red blood cells (see table 9).

might likewise neutralize the virus. That this was indeed the case was shown in the experiment of table 9 and text-figure 13. In this study a series of mixtures were made with virus (in filtered plasma) and heated anti-guinea-pig-tissue immune serum. A like series was prepared with the same immune serum absorbed with sheep red blood cells as described in the section on methods. The data given in table 9 are plotted in text-figure 13. It was evident that the absorbed serum was without effect on the virus, whereas the unabsorbed serum was strongly neutralizing, reducing the level of infectivity to the extent of 90 percent in this experiment. The results here give the impression that Forssman antibody, under the conditions with respect to the proportions of virus and antiserum, exhibits neutralizing capacity somewhat less than either antiviral or anti-chick-tissue immune rabbit serums. This appearance was not limited to the results of this experiment alone but was seen in other studies of this type.

f) Neutralization with serums from chickens immunized with the virus.—The experiments recorded thus far were made with immune serums from the rabbit, in which all of the viral antigens presumably were heterologous with respect to species. Under proper conditions, principally in relation to the amount of virus employed for immunization, highly effective antiviral immune bodies are readily produced in the chicken, the natural host of the agent. These antibodies, which are very active as neutralizing and precipitating reactants but which show no complement-fixing activity, have been of great value in the final analysis of the specific antigenic constitution of the virus. A brief description of the findings in this phase of the work has already been reported (*11*); it has seemed desirable, however, to include here, for purposes of continuity in the development of the problem, the supporting results of further studies with these serums.

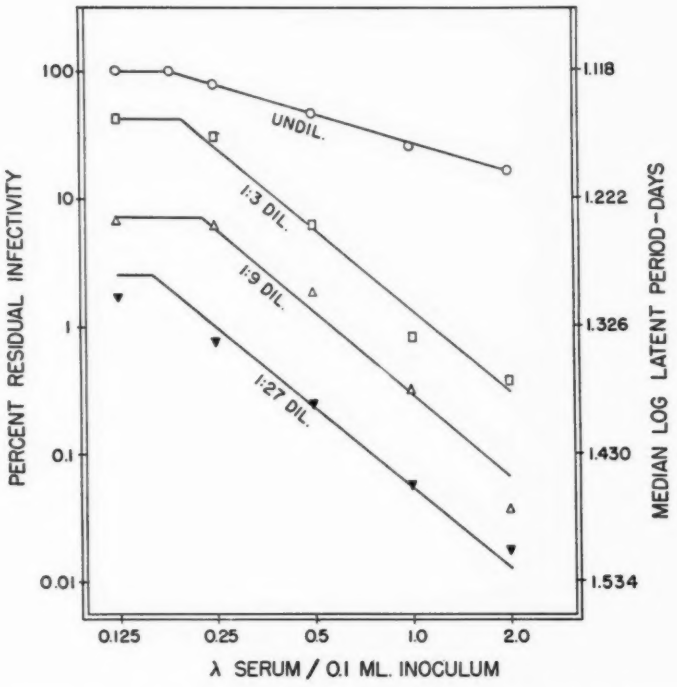
The protocol of an experiment in neutralization is recorded in table 10, and the data of the table are described graphically in text-figure 14. This was another checkerboard experiment like that of text-figure 8 with rabbit serum. The virus was present in the pooled, filtered plasma from several diseased birds and in 3 successive threefold dilutions of the plasma.

The antiserum was strongly neutralizing in very small amounts (compare with values of rabbit serum employed in the experiments already described). In some respects the pattern of neutralization observed with the hyperimmune chicken serum was like that occurring with the rabbits. One major difference was the slope of the relation of residual infectivity to serum dilution obtained with the largest amount of virus. Another difference was the relation between virus amount and serum dilution at which neutralization first became evident. With the rabbit serum, this relation varied and was essentially linear with virus amount. In the instance of the chicken serum, the break was independent of virus amount. The reasons for these differences have not been investigated.

TABLE 10.—*Neutralization of virus in successive dilutions of plasma with serum from a chicken hyperimmunized with concentrate*

Serum	Virus plasma* (λ)	No. particles† ($\times 10^8$)	Immune serum* (λ)	Chicks inoculated‡	Chicks positive	Median log latent period (*days*)	Residual infectivity§ (*percent*)
None	20	200	—	24	21	1. 114	120
Antiviral	20	200	2. 0	26	22	1. 204	16
"	"	"	1. 0	25	21	1. 183	26
"	"	"	0. 5	27	21	1. 157	46
"	"	"	0. 25	24	22	1. 132	79
"	"	"	0. 125	25	23	1. 121	102
None	6. 7	67	—	28	27	1. 156	46
Antiviral	6. 7	67	2. 0	27	18	1. 370	0. 4
"	"	"	1. 0	23	12	1. 333	0. 9
"	"	"	0. 5	27	21	1. 242	7. 0
"	"	"	0. 25	26	24	1. 170	33
"	"	"	0. 125	24	21	1. 164	38
"	"	"	0. 062	23	20	1. 150	55
None	2. 2	22	—	26	21	1. 226	9. 1
Antiviral	2. 2	22	2. 0	21	12	1. 470	0. 04
"	"	"	1. 0	23	13	1. 376	0. 3
"	"	"	0. 5	16	13	1. 299	1. 8
"	"	"	0. 25	23	21	1. 244	6. 0
"	"	"	0. 125	21	17	1. 237	7. 1
"	"	"	0. 062	24	18	1. 248	9. 1
None	0. 74	7	—	28	25	1. 237	6. 8
Antiviral	0. 74	7	2. 0	27	7	1. 503	0. 02
"	"	"	1. 0	27	7	1. 456	0. 05
"	"	"	0. 5	23	16	1. 388	0. 2
"	"	"	0. 25	24	17	1. 342	0. 7
"	"	"	0. 125	25	19	1. 303	1. 6
"	"	"	0. 062	24	17	1. 301	1. 7

*Volume per 0.1 ml. inoculum.
†Number of virus particles per inoculum.
‡Corrected number of chicks inoculated [see (17) for method of calculation].
§Calculated on the basis of latent-period relations.



TEXT-FIGURE 14.—Neutralization of virus in filtered plasma and in 3 successive threefold dilutions of the plasma by antiviral immune serum from the chicken (see table 10).

g) Precipitation of the virus with the serum of a chicken hyperimmunized with the agent.—The phenomenon of precipitation of the virus with hyperimmune chicken serum has been described briefly in an earlier report (*11*). Virus in sufficiently high concentration in plasma or concentrates may be precipitated quantitatively with optimum quantities of the immune serum. Within the limits of the accuracy of the methods of estimate employed, it was found that the virus particles and the enzyme activity were precipitated proportionally. In the present studies, the problem was to determine whether the normal chick-tissue and Forssman antigens followed or were separable from the virus particles in the precipitin reaction. The outcome of such inquiries is illustrated in tables 11 and 12.

In the experiment of table 11, hyperimmune chicken serum, 0.1 ml., was mixed in 3 different dilutions with 0.1 ml. of plasma of high virus content and 0.2 ml. of 15 percent NaCl solution. After incubation at 37° C. for 2 hours and standing overnight in the refrigerator, the preparations were spun at 2,000 × g for 10 minutes and the supernatant fluid was pipetted from the sediment. Estimates were made of the number of virus particles and of Forssman antigenicity remaining in the supernatant fluid. Forssman antigen was titrated against Forssman antibody induced

TABLE 11.—*Distribution of virus particles and Forssman antigen remaining in suspension after treatment of virus-containing filtered plasma with the serum from a chicken hyperimmunized with the virus*

Serum dilution	Particle count*		Forssman antigen	
	(× 10^9)	(*percent*)	(*titer*)	(*percent*)
Control	1, 150	100	159	100
Undiluted	340	30	50	31
1:4	1, 100	96	139	87
1:10	1, 100	96	172	108

*Per ml. of final mixture of plasma and immune serum.

in the rabbit with guinea-pig tissue. The data of table 11 show the remarkable correlation between the particles and Forssman antigen remaining in the supernatant fluid. Such studies were possible with the supernatant plasma because of the low content of Forssman antigen in plasma as noted earlier.

Studies of the relations of virus particles and normal chick tissue in the precipitin reactions were made with virus concentrate sedimented through 3 cycles of centrifugal fractionation. The concentrate was employed in order to avoid interference by the highly antigenic plasma proteins. Because of particle aggregation of repeatedly sedimented virus, estimates of the agent could not be made by particle count; instead, enzyme activity was used as the measure of virus amount. Mixtures were prepared of 0.1 ml. of virus concentrate; 0.1 ml. of various dilutions of immune serum, and 0.2 ml. of 15 percent NaCl. Incubation was carried out as described above, and the respective precipitates were collected by centrifugation at 2,000 × g for 10 minutes. The precipitates were then washed twice with successive volumes of 2 ml. of 8 percent NaCl solution. The results of the determinations of enzyme activity and of normal chick-tissue antigenicity of the precipitates, measured by titration against antinormal chick-tissue immune serum from the rabbit, are given in table 12. The data showed a fairly close correlation between the enzyme activity and chick-tissue antigenicity precipitated by the various quantities of serum. Although there is disparity between the absolute percentages of the two attributes in the respective precipitates, the relative values show a fairly good agreement. It would appear that, in each precipitate, the percent enzyme

TABLE 12.—*The relation of chick-tissue antigen to the virus enzyme activity in the precipitate occurring on treatment of a virus concentrate (obtained by 3 cycles of sedimentation) with the serum from a chicken hyperimmunized with the virus*

Serum dilution	Enzyme activity*		Chick-tissue antigen	
	(γP)	(*percent*)	(*titer*)	(*percent*)
Control	615	100	87	100
Undiluted	459	75	38	44
1:2	377	61	32	37
1:4	110	18	9	10

* γPhosphorus liberated by 1 ml. of final mixture of concentrate and immune serum.

activity was approximately twice that of the chick-tissue antigenicity. An explanation of the difference is not evident at the moment, though it is probable that it was related to the restricted serologic activity of the virus particles tightly coherent in the aggregated masses constituting the precipitates.

The nature of the material participating in the precipitin reaction and that of the precipitate, determined as previously described, is clearly illustrated in the electron micrographs of figures 4 to 7. An electron micrograph of untreated plasma containing 2.8×10^{11} particles per ml. is shown in figure 4. The initial stage of the formation of the precipitate which was obtained after 1 minute with 0.1 ml. of the plasma dialyzed overnight against 8 percent NaCl and then mixed with 0.1 ml. of immune serum and 0.1 ml. of 15 percent NaCl is seen in figure 5. The large agglomerates occurring after 3 minutes are shown in figure 6. Figure 7 is an enlargement of figure 6. The observation of principal significance in this electron micrographic study of the precipitin reaction was the visual evidence of the involvement of the particles which have been identified as the virus. In the enlarged picture, the individual particles can be seen in the precipitated masses. There is no evidence of material in the agglomerates extraneous to the virus particles. While these pictures are not submitted as quantitative evidence of the absence of nonviral substance, it is certain that any material other than the particles is not susceptible to portrayal by the method of electron micrography employed. It is of considerable interest to note also the lack of material in the background between the masses of particles.

Summation.—In this section it has been shown that the virus concentrates contained antigen capable of inducing antibodies in the rabbit which not only fixed complement with the concentrates as seen earlier, but effectively neutralized the specific infectious activity of the agent. This finding demonstrated conclusively the presence in the concentrates of the virus in sufficient amounts to act as effective specific antigen in the rabbit. It was now observed, further, that the immune serum induced in the rabbit by normal chick-tissue homogenate or by normal chicken serum exhibited the same property to neutralize the virus in reactions which did not differ qualitatively or quantitatively from those involving the specific antiviral immune bodies from the rabbit. In addition, homogenates of normal chick tissue were able to absorb both neutralizing and complement-fixing antibodies from the antiviral immune rabbit serum. If it can be assumed that neutralization signifies union of the immune serum with a specific constituent of the virus, the interpretation is inescapable that the host tissue antigen is not only a vital part of the virus particle but, also, of that part of the particle determining the property of infectivity. Similar conclusions are reached with respect to structural relations of Forssman antigen to the virus particle. The interpretations based on the neutralization experiments received full corroboratory support in the studies with chicken serum in the precipitation of virus particles, enzyme activity, chick-tissue antigen, and Forssman antigen.

One explanation which might be offered to account for the antiviral immune bodies induced in the rabbit with normal chick tissue is the possibility that the supposedly normal chick tissues and normal chick serums used as antigen may have contained virus in the absence of noticeable disease. Obviously, complete control of this hypothetical factor would be impossible except, perhaps, with chicks reared aseptically. Even in this case, however, there would remain the hazard of virus transmission through the egg. Nevertheless, contribution by occult virus can be ruled out for practical purposes. The antibodies were produced by the tissue of every normal bird studied; the immune serums from the rabbit as well as those from mice were not pooled but were studied separately. The experiments cited have been selected for illustration of the principles described from the many made during the past 3 years. Neutralization by Forssman antibody produced by guinea-pig tissue provides indirect evidence of the validity of the interpretation of the results obtained with normal chick-tissue immune serum from the rabbit. In this respect, the possibility would seem remote that avian leukosis virus should be indigenous to the guinea-pig population.

The experiments provided no basis for distinguishing between the complement-fixing and neutralizing antibodies produced by the virus in rabbits. Although both antibodies were absorbed by normal chick tissue in approximately proportional amounts, the experiments were not of a nature permitting final statements. Many problems of the antigenic constitution of the agent and of the complex serologic reactions remain for further investigation. The experiments described, though quantitative in design, have been made principally for the purpose of demonstrating qualitative aspects of the antigenic constitution of the myeloblastic leukosis virus.

Discussion

The findings described here appear to establish the occurrence of materials indistinguishable from chicken-tissue and from heterophile antigen, which induces and reacts with Forssman antibody, as intrinsic or integral constituents of the virus of avian erythromyeloblastic leukosis. With the present understanding of the nature of the disease caused by the agent under study, these interpretations may be regarded as applicable specifically to the agent responsible for *myeloblastic leukosis or myeloblastosis*. It may be concluded further, from the data of the various tests, that the antigens are essential parts of the portion of the virus particle responsible for its infectious properties. Interpretations based on the combined results obtained with both rabbit and chicken immune serums indicate a minimal complexity related to at least 3 antigens constituting the radical or substance of the particle concerned with infectivity. It may be assumed that the total virus particle consists of material foreign to the rabbit and that each different antigen would excite in this animal its respective antibody. The tests revealed two of these antibodies, the one related to chick-tissue antigen as such and the other to the Forssman antigen, both of which could be detected specifically

by virtue of their neutralizing activities. No definite evidence of an antigen other than the 2 mentioned above was obtained with the immune serums from the rabbit. On the contrary, however, it may be assumed, though it is by no means certain, that neither chick-tissue nor Forssman antigen derived from or similar to chick derivatives would produce antibodies in the homologous host. It was seen, nevertheless, that the virus concentrates did contain antigen capable of producing antibodies in the chicken which strongly neutralized and precipitated the virus particles. This evidence points to the third antigen, peculiar to the virus, which, together with the normal chick-tissue and Forssman antigens, constitutes, at least in part, the infectious moiety of the agent. The results of these experiments have been so strikingly clear that it would appear entirely remote to consider possible mechanisms of adsorption as significant contributors to the phenomena. Instead, with the present concepts of immunologic specificity, there seems no escape from the interpretation that the virus particles contain, as vital constituents, materials which are either host material incorporated in the particles or are components synthesized concurrently within the synchronized processes resulting in the formation of the particles.

The concept that some viruses should or that some do contain the equivalent of host tissue components is by no means new. There are many (*30–34*) who have considered the possibility that viruses may have originated initially as derivatives of one sort or another of the components of their respective host cells. The evidence against this hypothesis is preponderantly negative; namely, there has been little to show any evidence of kinship between virus and host, since autonomy and specificity have been characterizing attributes of the agents. Nevertheless, the concept has not been without some experimental support. This came first in reports of the association of host antigen with viruses responsible for avian neoplasms, which are often quoted in support of the hypothesis of the intrinsic origin of viruses. The work in the field prior to 1934 was reviewed by Foulds (*35*), who remarked on the confusion in the interpretations of the serologic studies. Further investigations were made by Amies (*36*) and Amies and Carr (*37*), who concluded that the virus of chicken sarcoma I contained host tissue. Furth and Kabat (*38–40*), in their studies with chicken sarcoma and leukosis, demonstrated normal chick components in their virus concentrates but regarded them (*41*) as host tissues sedimented with the virus. The outstanding earlier work in this field was that of Knight (*42*), who demonstrated an intimate, if not essential, relationship of analogues of host components to the virus of influenza by means of the precipitin reaction occurring with virus in the presence of antinormal chick-antigen immune serums. Neutralization by such serums was not demonstrable. Prior to this, Cohen (*43*) had found normal host and Forssman antigens associated with concentrated influenza virus, but his interpretations were not clear.

It would be futile to attempt to reconcile the present results with any of the earlier findings except those with the influenza virus. There have

been no other investigations with the virus of myeloblastosis, and it is possible that its properties are unique. A probable basis for much of the variation previously seen was the failure to give full consideration to the quantitative factors related to the physical mass of the respective viruses in the preparations employed, a matter recently discussed by Bryan (*44, 45*). From the reports of other investigators and experience in this laboratory, it is certain that these factors, as they pertain to myeloblastosis, are entirely different from those concerned with the viruses of other avian tumors. Myeloblastosis was an extraordinarily fortunate choice for study because of the high concentration of virus in a medium as simple as plasma. In recent experiments (*46*) with the sister leukemia, erythroblastosis (which was one of the conditions studied by Furth and Kabat), there is scarcely enough virus for identification, and it is certain that very little could be obtained by centrifugation of practical volumes of plasma. There has been no evidence of an abundance of virus in solid tumors, from which separation of agent is very difficult, as the lack of results in this field have indicated. The factor of mass is of importance not only in physical and chemical studies but also in the production of antibodies and in serologic reactions. With the large quantities of myeloblastic leukosis virus available, the production of antibody and the demonstration of reaction have been no more difficult than with many other viral agents. A serious impediment for the earlier work was the lack of the means for accurate titration of infectivity (*47*), which has been developed only recently for myeloblastosis (*17*) and for the Rous sarcoma virus (*48*).

Knight's observations with the influenza virus are a different matter. The virus was plentiful in preparations fully characterized by physicochemical methods. His system, however, was not susceptible to the application of the final specific criteria, since the antinormal chick-immune serum failed to neutralize the agent. The results did locate normal chick and mouse antigens in the purified preparations in a form inseparable from the influenza virus particles but did not eliminate the possibility that the antigens were adsorbed on the virus. The failure of the antinormal chick-immune serums to neutralize, though precipitating the virus, is by no means positive evidence of the absence of host antigen from the virus particles. It is entirely possible that the host antigens may exist in the influenza virus particle apart from the center responsible for infectivity.

It has become increasingly apparent that great caution must be exercised in generalization of interpretations from one virus to another. In essence, studies of viral properties have revealed more differences than similarities among the agents. It can be said that most of the criteria applicable to the problem of the relationship of host tissue to virus have been employed in the study of the agent of myeloblastosis. The results give considerable weight to the hypothesis that this virus, at least, may well have assumed existence in the beginning as a derivative or product of its specific host cell, the progenitor of the myeloblast, or of the myelo-

blast *per se*. Though, in the light of the generality of knowledge of viral specificity, the hypothesis is not too attractive, it seems a much more reasonable view than one necessitating an explanation of the manner in which an agent originating extrinsically could assume the structure of its host cell. All the findings, including the enzymatic activity to dephosphorylate adenosinetriphosphate, fit neatly into the pattern ideal for the nearly perfect state of symbiosis essential for the perpetual existence and increase of both parasite and host cell. The process terminates in the chicken, not by mishap to the immediate relationship between the virus and its cell, but by death of the host no longer able to support the prodigal activity. While there is no justifiable reason to extend the interpretation beyond this one agent, it is still possible to regard the findings with the virus of myeloblastosis as at least a partial template and a substantial basis for further investigations with other agents, especially those responsible for other forms of neoplasia.

It has been stated before that the original purpose of these studies was to obtain information about the state of homogeneity of the purified virus concentrates. The results bear significantly on this problem. Repeated observations throughout the course of the work with the virus of myeloblastosis have failed to reveal more than one population of particles of characteristic appearance in electron micrographs. This is not meant to imply that other substances of a size within the resolving power of the electron microscope have not been seen. On the contrary, amorphous materials of many sizes in addition to the virus particles occur in pictures of various plasmas. The relative proportions of these substances and particles vary greatly. In plasmas containing few particles, the extraneous material predominates, but in those of high content of virus particles, the latter greatly overshadow the extraneous material. It would seem that the material other than the virus particles occurs in similar amounts in all plasmas, and much of it is lost in the purification process. If the elimination of the extraneous material is approximately the same for each plasma, it might be expected that the proportion of virus particles to nonviral substances would be of the order of a 1,000-fold greater in plasmas of 10^{12} particle content than in those of 10^{9}. This likelihood is substantiated to a large extent by the obviously high proportion of virus particles to extraneous material in the concentrates from good plasmas. A good impression is given in the precipitin procedure with antiviral chicken immune serum. When serum is added to very turbid virus suspensions, there remains, after precipitation, a water-clear supernatant fluid. That some amorphous material remains in the supernatant fluid is demonstrable by electron micrography, but it is quite evident that the amount is negligible in comparison with that of the specifically precipitated particles. The quantitative proportions of virus and extraneous material are subject to investigation with ultramicroanalysis, which will be a further objective of the work. An important criterion of homogeneity was applied in the electrophoretic experiments. The single, sharp boundary, though small, constituted good evidence of the lack of

large proportions of material extraneous to the virus particles. The serologic observations eliminate, conclusively, the need for postulating extraneous material to account for host and Forssman antigenicity, since all of the phenomena are explicable as related directly to the complexity of the virus particle. Actually, the interpretation may be extended to include the positive evidence of the essentially quantitative interrelationships of particles, enzyme activity, and serologic behavior. While the data obtained by these correlations may not be acceptable as of high precision, it is, nevertheless, certain that no evidence existed of material other than the virus particles.

Summary

Studies have been made of the antigenic constitution of concentrates of the virus of erythromyeloblastic (myeloblastic) leukosis obtained by ultracentrifugal fractionation of the plasmas of birds with the disease. It has been found that such concentrates, repeatedly fractionated in 3 to 5 cycles of ultracentrifugal sedimentation, contain the particles characteristic of the virus and, in addition, materials exhibiting the serologic behavior of normal chick-tissue and Forssman antigens, as determined by direct and cross complement fixation and Forssman reactions. The antigens followed quantitatively the virus particles through the fractionation procedure, and physical studies demonstrated that the sedimentation and electrophoretic properties of the antigenic materials could not be distinguished from those of the virus particles. It could be inferred, therefore, that the material responsible for chick-tissue and Forssman antigenicity was a particulate entity, or entities, which could not be differentiated physically from the virus particles. The absence of any electron microscopic evidence of more than one population of particles in the virus concentrates strongly suggested that the antigenic materials were intimately associated with the virus particles either adsorbed on or, possibly, as specific constituents of the particles.

With the use of immune serums produced in the rabbit and in the chicken, specific tests were made to determine the relationships of the antigens to the virus particles. It was observed that antiviral immune bodies from either the rabbit or the chicken neutralized virus infectivity. Immune serums produced in the rabbit with homogenates of normal chick tissue and with normal chick serum were likewise fully effective in neutralization of the agent. Neutralization of somewhat less efficiency was observed with Forssman antibody induced in the rabbit with homogenates of normal guinea-pig kidney. The neutralizing activity, as well as the complement-fixing capacity, of antiviral immune serums from the rabbit was absorbed by normal chick tissue. In like manner the virus-neutralizing capacity of Forssman antibody was eliminated by absorption of Forssman antiserum with sheep red blood cells. The virus particles present in plasma or concentrates were precipitated by the specific antiviral immune serum from the chicken. With the virus particles there was precipitated, in proportion, the material responsible for normal chick-tissue and Forssman antigenicity. The specificity of virus-particle

precipitation with chicken immune serum was documented by electron micrographs.

From the results of these experiments, it has been concluded that material indistinguishable antigenically from normal chick (host) tissue and Forssman antigen are vital, intrinsic constitutional elements of the virus of avian myeloblastosis. It is evident, in addition, that the antigenic components are integral parts of that portion of the virus particles exerting the property to transmit the disease. The involvement of the antigens in the processes of neutralization eliminates the possibility that the phenomena are related to simple adsorption of the antigens on the virus particles.

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PLATE 44

FIGURE 1.—Virus concentrate obtained with a single cycle of centrifugal fractionation illustrating the slight aggregation of the particles characteristic of sueh preparations from plasmas of high virus content (see fig. 4, which shows the state of the particles as they appear in plasma). × 13,800

FIGURE 2.—Virus concentrate after 2 cycles of centrifugal fractionation, showing increased aggregation of the particles. × 13,800

FIGURE 3.—A third-cycle concentrate illustrating aggregation of the particles in such preparations. × 13,800

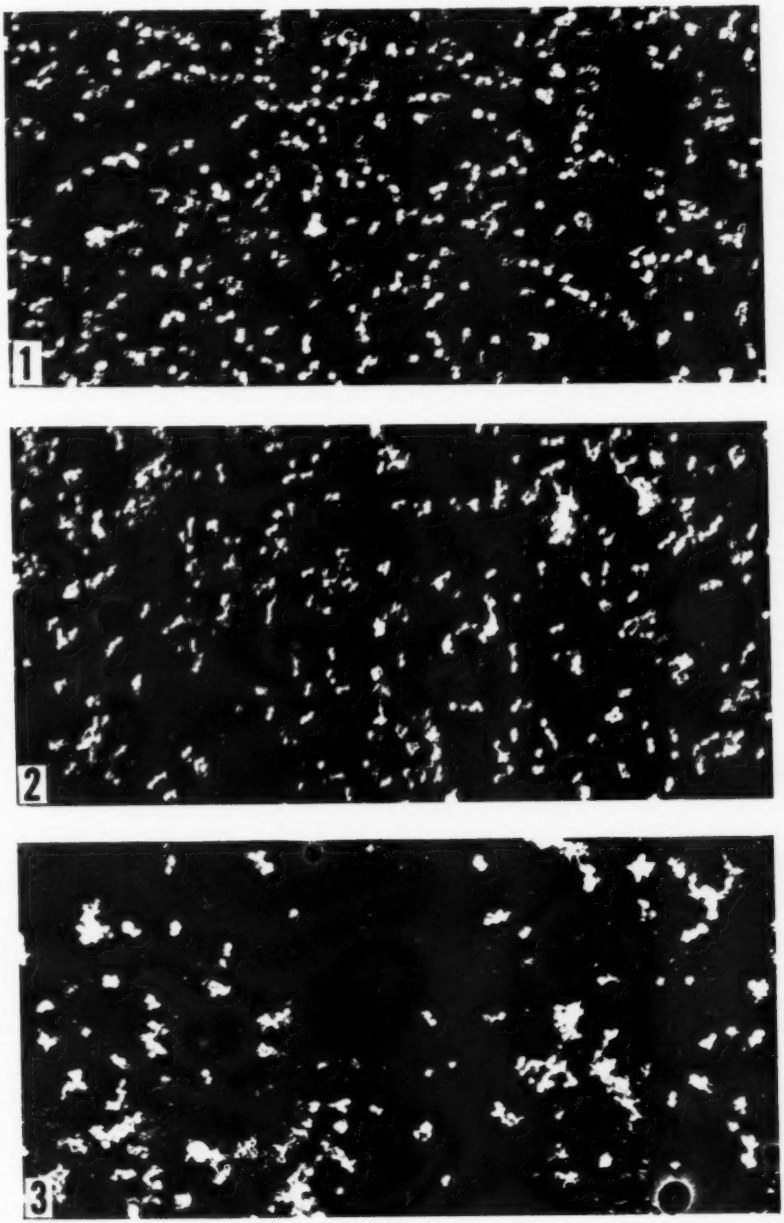

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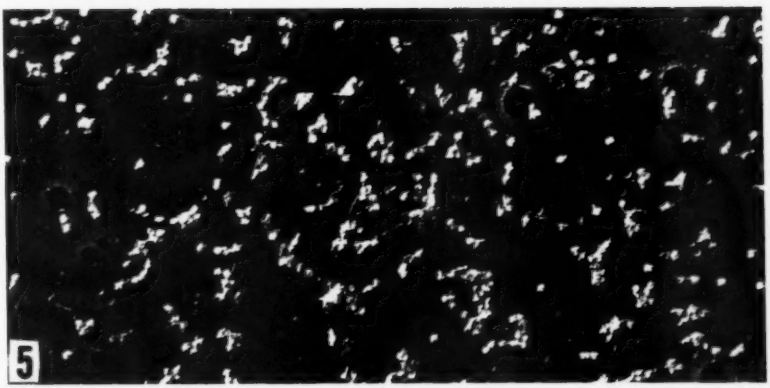
PLATE 45

FIGURE 4.—Virus particles in filtered plasma. × 13,800

FIGURE 5.—Specific aggregation of the particles of the plasma of figure 4 occurring within 1 minute after treatment with antiviral immune serum from the chicken. × 13,800

FIGURE 6.—Specific aggregation of the particles of the plasma of figure 4 occurring within 3 minutes after treatment with antiviral immune serum from the chicken. × 13,800



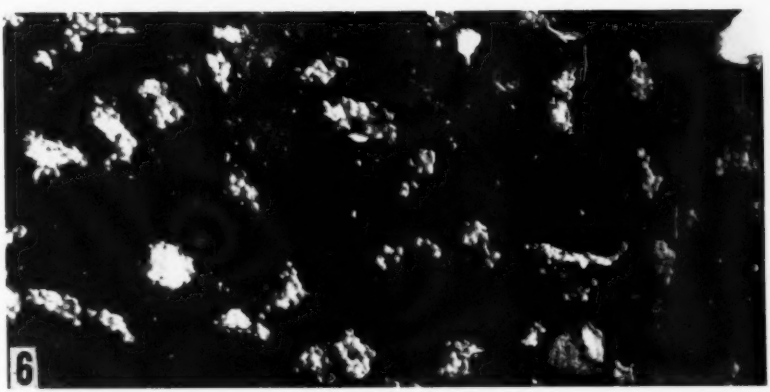


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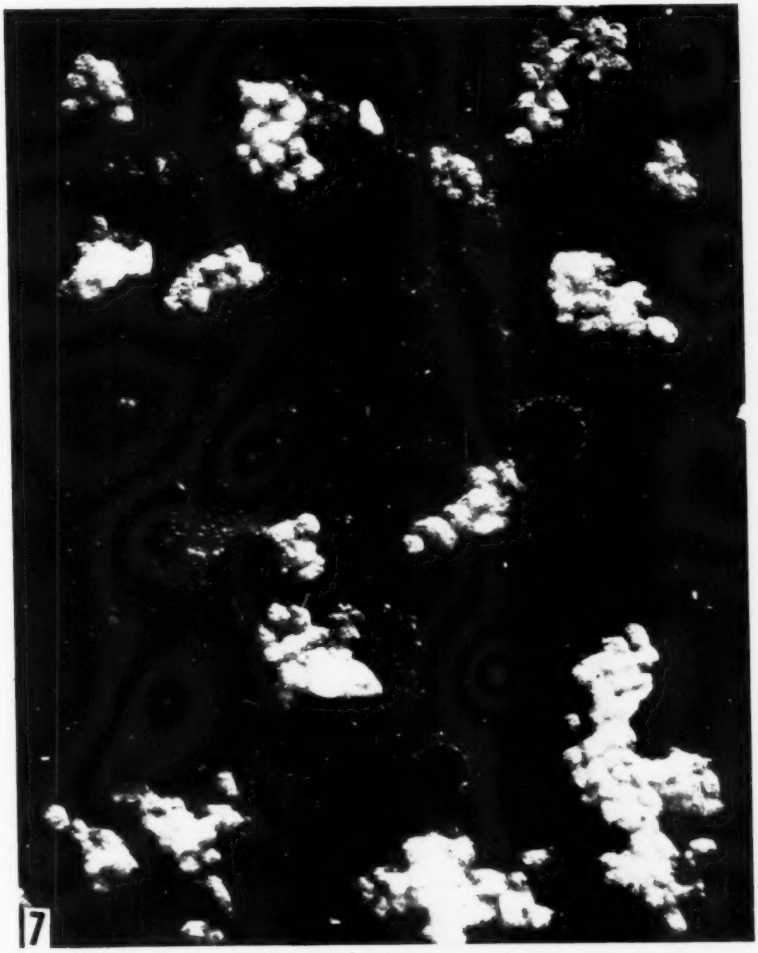
PLATE 46

FIGURE 7.—An enlargement of the electron micrograph of figure 6. × 34,000

FIGURE 8.—Electrophoretic diagram of the migrating boundary of the virus particles observed in experiment 3, *p*H 7.0, table 3. The photograph shows the ascending boundary just after it passed the junction of the upper and lower anode sections of the divided Tiselius cell.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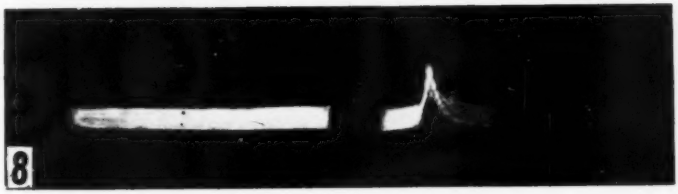
8

Cancer-Cell Seeding of Operative Wounds [1]

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At present the cure of cancer depends upon the complete destruction or removal of all cancer tissue from the body. This can be accomplished only by complete surgical removal, destruction by ionizing radiation, or chemical cautery. Chemotherapy, as well as the multiple forms of endocrine control, seems to be a purely palliative means of controlling cancer at present. These crude methods of therapy, whether they be for palliation or cure, continue to be the only effective ones. It is, therefore, important to discover why present-day methods of treatment fail. One cause of failure is the seeding of operative wounds at the time of definitive surgery. It is this problem that we wish to present in this paper.

Incomplete surgery will, of course, lead to failure of therapy. The surgeon may transect the cancer, leaving a portion of it attached to the wall of the operative wound, and this will lead to regrowth of tumor from that focus. It is also possible for clumps of tumor cells to be implanted or "seeded" in an operative wound, causing regrowth of small bits of tumor. This sometimes occurs when lymph and other tissue fluids are extruded into the wound at the time of surgery. The necessary trauma and pressure on the tumor associated with the transection of lymphatics would undoubtedly cause small clumps of individual cells of tumor tissue present in lymphatics to break off and be extruded into the wound. Once these tumor emboli are free in the wound, they become embedded in small clots or fragments of cut muscle which may supply an excellent medium for the tumor to grow and attach itself to the local area. Exfoliated cells from the surface of ulcerated tumors may also act as foci for regrowth of cancer.

The finding of clumps of tumor cells in operative wounds, on instruments, in wash water, on surgeon's gloves, etc., is not a new observation. For a long time pathologists have been able to demonstrate cancer cells on instruments, along aspiration biopsy tracts, and in operative wounds away from the primary site of the tumor (*1–4*). Ackerman and Wheat speak of the "implantation of cancer—an avoidable surgical risk?" (*5*).

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

358499—55——6

That tumor emboli break off and are free in body fluids was demonstrated by the work of Fisher (*6*), who found tumor cells in blood of the inferior mesenteric vein during colon resections in 8 of 25 patients studied. In head and neck, breast, or other cancers in which *en bloc* surgical resection is the usual method of therapy, this same mechanism occurs and may possibly be an important factor in the large number of treatment failures. This is indicated in table 1 which summarizes reports in the literature by several authors. The data show that in head and neck cancer, local recurrence develops in about 40 percent of cases and that in breast, stomach, and colon cancer local recurrence after surgical removal is a common cause of failure of therapy. If such complications are caused by local implantation of cancer cells in wounds and suture lines, it should be possible to demonstrate the presence of these cells in wound washings. The present study was undertaken to demonstrate that malignant tumor tissue may be found in washings from operative wounds following *en bloc* surgical removal of cancer.

Methods and Materials

Just before the closure of the skin flaps following surgical removal of a primary cancer and its lymph-node metastases, in cases of primary operable but advanced head and neck, breast, or pelvic cancer, the wound created by such a surgical procedure is thoroughly washed with sterile physiologic-saline solution, using a fine spray. The washings are collected in a glass container and mixed as quickly as possible with a fixative of 70 or 95 percent ethyl alcohol. The washings are then centrifuged at 2,500 r.p.m. for 10 minutes. Part of the sediment is covered with 10 percent formalin, and the button of fixed sediment is embedded in paraffin and sectioned in the routine manner for tissue sections. Another part of the sediment is smeared directly on clean glass slides, and these slides are immersed in a solution of equal parts of 95 percent ethyl alcohol and ethyl ether. The slides are allowed to remain in the fixative for 30 minutes. While still wet they are stained by any routine nuclear and cytoplasmic stain. Slides to be saved for special staining procedures may be kept in alcohol and ether fixative indefinitely. The Papanicolaou staining technique is the standard stain used in this laboratory. After the staining is completed, slides are promptly mounted, using a permanent type of seal. Routinely these slides are screened by technicians trained to interpret exfoliative cytologic material, and cells or groups of cells to be studied by the pathologist are marked by the technician.

Results

Wound washings from 36 cases of locally operable cancer at various sites were studied, with results as outlined in table 2. In 10 instances there was definite evidence of tumor tissue in the wound washings and in 5 instances the wound washings were considered to contain cells or groups of cells of a suspicious nature but not definitely malignant. In 22 cases the wound washings were considered to show no evidence of residual tumor. The distribution of types of cases is shown in table 3.

TABLE 1.—*Reported incidences of local recurrences after surgery*

Site of cancer	Author	Cases (*number*)	Recurrences in operative area (*percent*)	Follow-up period (*years*)
Head and neck	Harrold (*7*)	204	44	3
	Beahrs (*8*)	121	17 percent living—an additional 28 percent died. Site of recurrence not given	
	Smith (unpublished data) (*9*)	31	45	2–3
Breast	Haagensen and Stout (*10*)	604	9.7—cases with disease limited to breast 31.2—cases with axillary nodes involved	5+
	Lewis and Rienhoff (*11*)	447	32.2	5+
	White (*12*)		10.8—cases with no axillary metastases 31.5—cases with axillary metastases	
	Hare (*13*)	165 (61 followed)	25	5
Stomach	McNeer (*14*)	92	46—in gastric remnant 9—duodenal stump 19—perigastric nodes and gastric bed	Autopsied cases that had subtotal gastrectomy
Colon	Cole (*15*)	55	16—in colon only—no. of cases with other local metastases not given	5

TABLE 2.—*Results of wound washings*

Case No.	Primary site of cancer	Type of operation	Local metastases	Results of wound washings	Follow-up
1	Tongue	Tongue and neck dissection	Yes	Negative	N. E. D.*—21 mos.
2	Larynx	Larynx and neck (post rad.)	Yes	Negative	N. E. D.—18 mos.
3	Tongue	Tongue and neck dissection	Yes	Negative	Metas. in other side of neck—12 mos.
4	Breast	Radical mastectomy	No	Negative	N. E. D.—15 mos.
5	Breast	Radical mastectomy	No	Negative	N. E. D.—15 mos.
6	Lip	Lip and upper neck dissection	No	Negative	N. E. D.—12 mos.
7	Skin of thigh (melanoma)	Neck and radical groin resection	Yes	Negative	Died—10 mos. Local and distant metastases.
8	Antrum Antrum	Radical antrum and radical neck dissect.	Yes Yes	Positive Negative	N. E. D.—11 mos. N. E. D.—1 mo.
9	Antrum (recurrent; post radiation)	Radical resection	No	Suspicious	Recurrence in wound—1 mo., excised—died—8 mos. Distant metastases.
10	Colon	Right colon resection	Yes (1 marg. node)	Negative	N. E. D.—10 mos.
11	Tonsil	Radical neck dissection; X ray to primary	Yes	Negative	N. E. D.—9 mos.
12	Scapula (chondrosarcoma)	Interscapulo-thoracic amputation	No	Positive	N. E. D.—9 mos.
13	Cervix	Pelvic exenteration	Yes	Negative	N. E. D.—8 mos.
14	Skin of ear (basal cell; invading bone)	Radical resection ear	No	Positive	N. E. D.—7 mos.
15	Posterior pharyngeal wall	Radical resect. in contin. with neck dissect.	Yes	Suspicious	N. E. D.—7 mos.
16	Cervix	Rad. hysterectomy and node dissection	No	Positive	Recurrence—2 mos. Resected—N. E. D.—5 mos.
17	Abdom. wall (implants from colon)	Resection abdominal wall	No	Negative	Recurrence—6 mos.
18	Ethmoid bones	Radical resection paranasal sinuses	No	Positive	N. E. D.—6 mos.
19	Soft tissue of leg (fibrosarcoma)	Wide local excision	No	Negative	N. E. D.—6 mos.
20	Abdom. wall (implants from colon)	Resection abdominal wall	No	Negative	N. E. D.—6 mos.
21	Tonsil	X ray and radical neck dissection	Yes	Negative	N. E. D.—6 mos.
22	Floor of mouth	Radical resect. floor of mouth in contin. with neck dissection	Yes	Suspicious	N. E. D.—5 mos.

23	Abdom. wall (desmoid)	Radical resection abdominal wall	No	Suspicious	N. E. D.—5 mos.
24	Skin of arm (melanoma)	Radical resection skin of chest wall	Yes	Negative	Died—2 mos. Dissem. disease.
25	Floor of mouth	Resection floor of mouth and radical neck dissection	Yes	Negative	N. E. D.—4 mos.
26	Cervix	Rad. hysterectomy and node dissection	No	Negative	N. E. D.—4 mos.
27	Cervix	Pelvic exenteration	Yes	Suspicious	Metas. to other areas—3 mos. Local?
28	Tongue	Resection tongue and neck dissection	Yes	Positive	N. E. D.—3 mos. Metas. to other side of neck—4 mos.
29	Cervix	Radical hysterectomy and node dissect.	No	Negative	N. E. D.—2 mos.
30	Breast	Radical mastectomy	Yes	Positive	N. E. D.—2 mos.
31	Breast	Radical mastectomy	Yes	Negative	N. E. D.—1 mo.
32	Alveolar ridge	Radical resection face	Yes	Positive	Recurrence—2 wks.
33	Cervix	Pelvic exenteration	Yes	Positive	Died—10 days P. O.
34	Skin of back (melanoma)	Radical resection and axillary dissection	No	Positive	N. E. D.—1 mo.
35	Tonsil	Radical neck dissection and X ray	Yes	Negative	N. E. D.—1 mo.
36	Colon	Left colectomy	Yes (1 marginal)	Negative	N. E. D.—1 mo.

*N.E.D.=no evidence of disease.

TABLE 3.—*Results of cytologic study of wound washings*

Site or type of cancer	No. of cases	Washings positive for cancer cells	Washings containing suspicious cancer cells	Local recurrences when washings were:		
				Pos.	Susp.	Neg.
Head & neck	16	5	3	1	1	
Cervix & colon	8	2	1	1		
Abdominal wall	3		1			1
Breast	4	1				
Melanoma	3	1				1
Soft part sarcoma	2	1				
Total	36	10	5	2	1	2

In the short time since this study has been under way, two of the cases in which the wound washings were *positive* have already developed recurrent tumor in the operative site. In an additional two cases in which no definite evidence of cancer cells was found in the wound washings, local recurrences of the tumor have developed. Finding clumps of tumor cells in 27 percent of this small series of cases certainly makes one suspect that this figure represents a minimum number of cases that could be expected to demonstrate local recurrence of tumors. It is reasonable to believe that in a number of cases the tumor cells might be free in the wound in such minute quantity that the chance of demonstrating them would be quite small. It should also be remembered that not all tumor cells contaminating a wound will grow, so that not every case with a positive washing will develop recurrences.

Discussion

The identification of malignant cells in the wound washings did not present too difficult a problem. Most of the washings examined contained small fragments of tumor tissue which could be readily identified (figs. 1, 2, 4). Occasionally a washing contained rather large fragments of tumor tissue, as in the case of one of the pelvic washings after hysterectomy (fig. 3, case 16). In this case a large fragment of tumor represented a biopsy from an epidermoid carcinoma and included epithelial pearl formation and abnormal mitoses as well as other bizarre tumor cells.

In all instances, cells or clumps of cells seen in specimens of wound washings were compared with tissue biopsies from the surgical specimen. In all the washings considered positive, the tumor cells identified corresponded in morphologic characteristics to tumor cells seen in the tissue sections. In one case of adenocystic carcinoma of the minor salivary gland, in which there was a mixture of glandular tumor tissue and more solid squamous-like tumor, both types of tumor tissue were identified in the washings from the surgical wounds (figs. 1 and 2).

An interesting case of basal-cell carcinoma involving the skin of the right external auditory canal as well as the mastoid bone provided not only a positive washing containing tumor cells (fig. 5, case 14) but also provided an unexpected fragment of tissue from the parotid gland, which

had been partially resected during the surgical procedure. This fragment was well preserved (fig. 6), and zymogen granules as well as the orderly glandular pattern, could be readily identified.

The primary difficulty in positive identification of tumor cells occurred in those washings in which numerous large phagocytes were found. In the absence of actual clumps of tumor cells, it was sometimes difficult to differentiate phagocytes from cancer cells. Because of this difficulty and because of the associated deterioration of cellular material in most of these washings, an attitude of skepticism and caution was adopted. Most of these washings were considered to be only suspicious, or even negative in some instances, even though the surgical procedure indicated that in all likelihood tumor fragments or cells should be present.

After the experience of examining numerous wound washings (both negative and positive) for tumor cells, it is now considered that in most instances in which tumor remains in the surgical wound or has been carried into the wound, these cells can be identified if washed out. Experience to date also seems to indicate that when tumor fragments are identified in wound washings there is a strong possibility of local recurrence of the tumor in the operative site. The number of tumor clumps present in the operative wound may be reduced by gentle, nontraumatic surgery, the use of multiple instruments, and, of course, by obtaining an adequate margin from all limits of the tumor at the time of surgery. When all these practices are observed, however, therapy in a significant number of cancer cases will fail because of the continued presence of tumor cells in the operative wounds. The trauma necessary to remove a tumor from the operative area, along with the necessary transection of lymphatic and blood vessels, will allow the ever-present tumor emboli to break off and be extruded into the operative wound. Studies are under way to develop means of destroying the tumor tissue which may be present in an operative wound. Effective local chemotherapy of cancer can best be accomplished at the time of the primary surgical removal of the cancerous tissue. If and when an effective chemotherapeutic agent is discovered which can selectively destroy cancer cells that either become embedded in the wound or break off and are free in the intact blood of lymph channels, effective therapy of cancer will be available.

Summary

Washings taken from completed operative wounds of 36 cases of advanced but locally operable cancers have been submitted to cytologic study. Of the 36 cases, positive identification of tumor cells was made in 10 cases, and in an additional 5 the washings were suspected of containing malignant tumor. The cytologic technique used is described. It is suggested that the presence of tumor emboli in such a large number of cases should point to this phase of therapy as a logical place to apply chemotherapy.

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Plate 47

Figure 1.—Fragment of tumor tissue in washings from the surgical wound, maxillary sinus area. × 950

Figure 2.—Fragment of tumor showing more squamous-like tumor cells in washings of maxillary sinus area. × 780

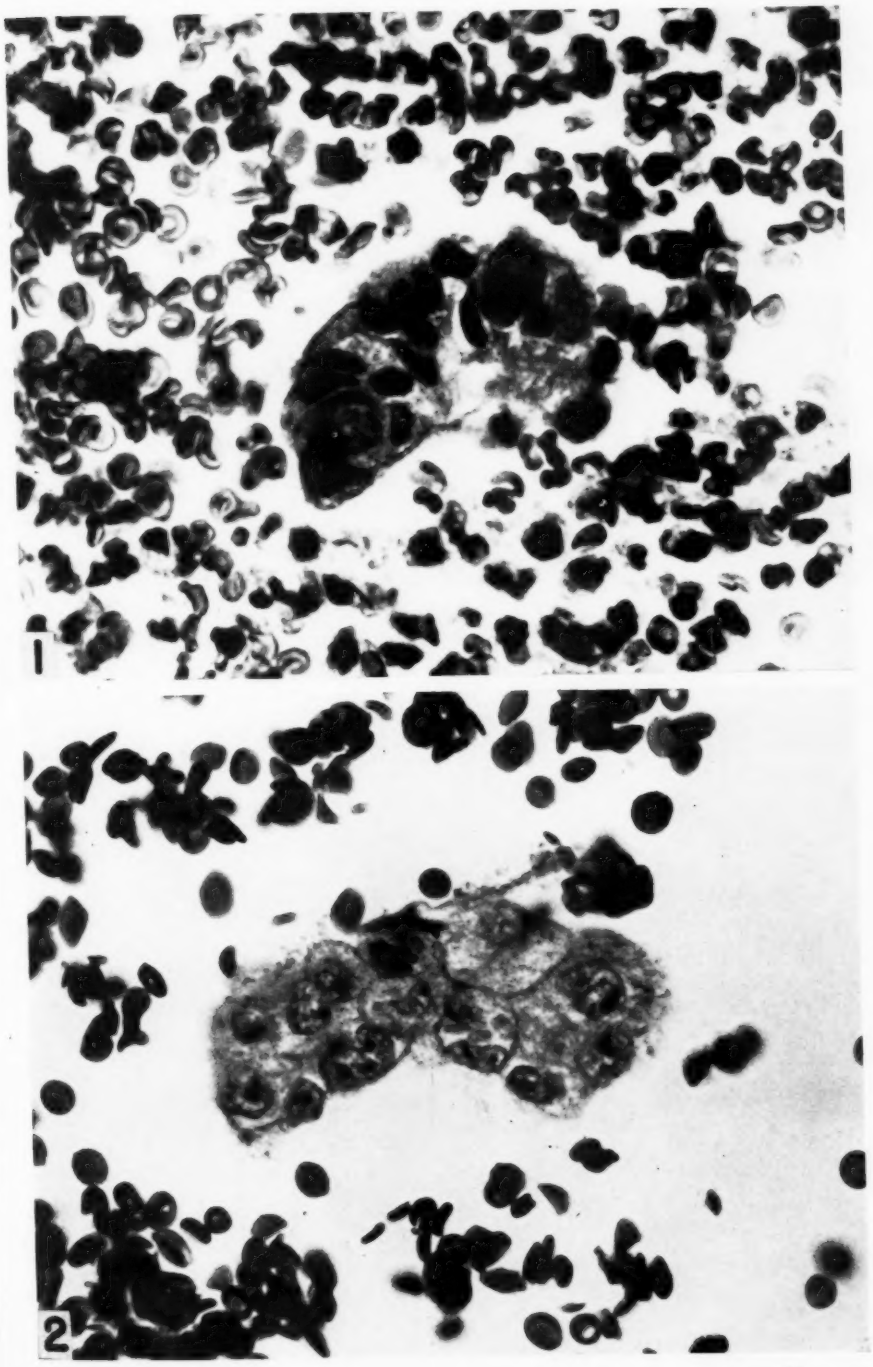
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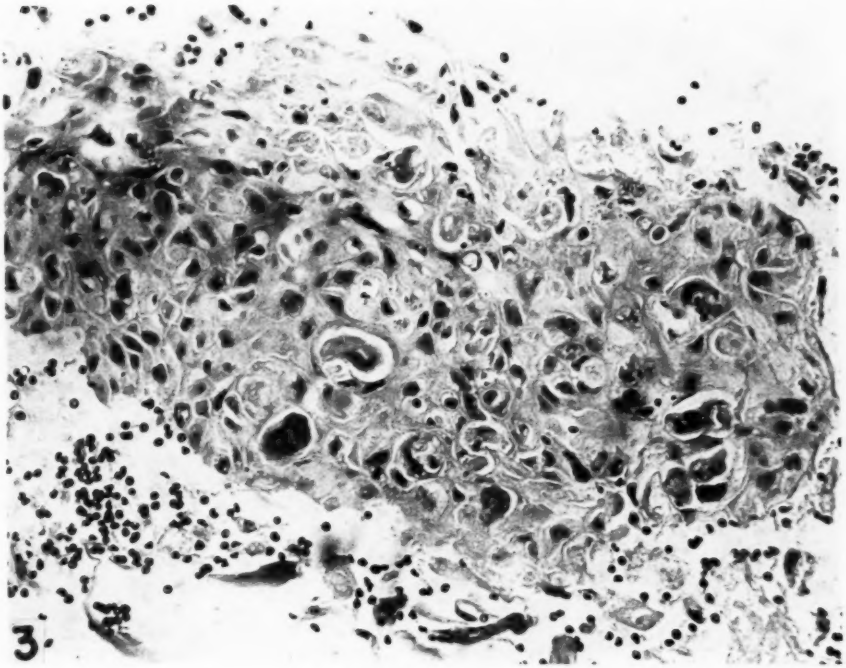
PLATE 48

FIGURE 3.—Fragment of epidermoid carcinoma in washings of pelvic area. × 210

FIGURE 4.—Clump of epidermoid carcinoma cells with "pearl" formation in washings of maxillary sinus wound area. × 290



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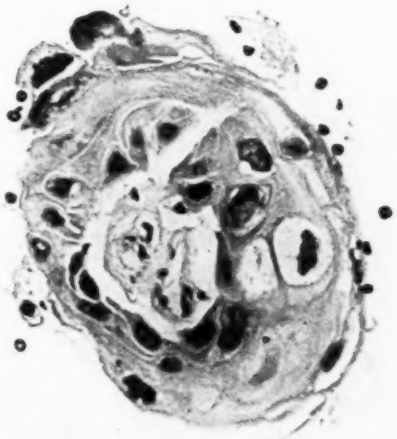

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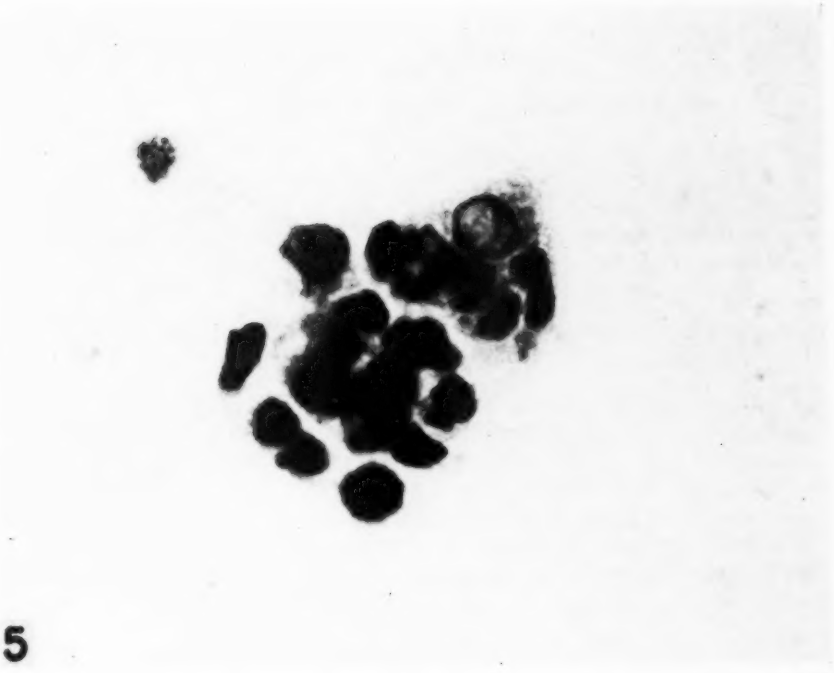
PLATE 49

FIGURE 5.—Clump of malignant epithelial cells in washings from surgical wound in preauricular area. × 950

FIGURE 6.—Fragments of normal parotid-gland tissue in washings from preauricular area. × 380



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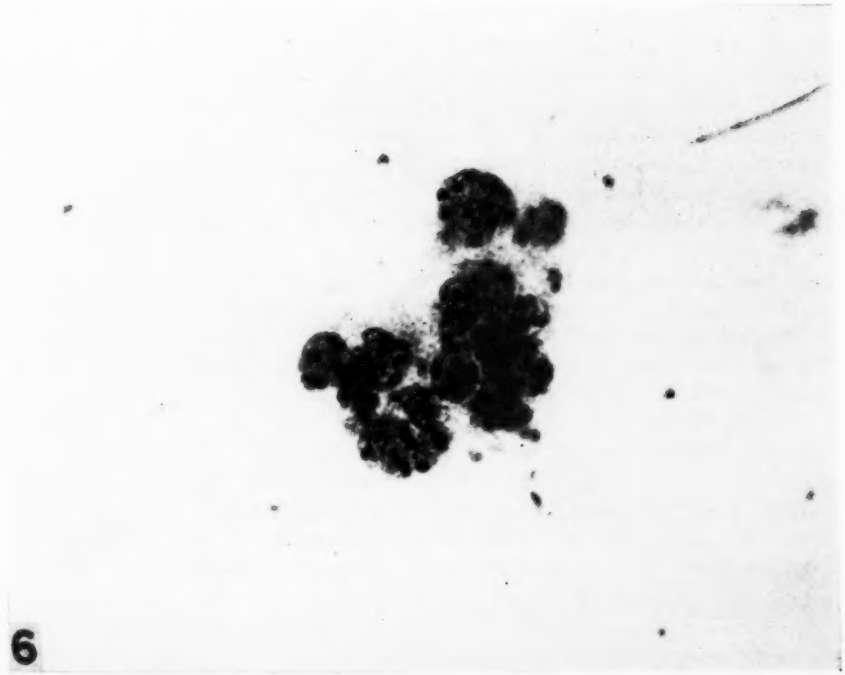

6

Intramandibular Tumors in Mice[1]

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In examinations of old mice in our laboratory an occasional swelling of the mandible was found (fig. 1). Further investigation disclosed that in some cases the swelling was caused by a cyst (fig. 2) and in others by a neoplastic growth (fig. 3). Similar tumors in mice were described by Zegarelli (*1*), who had examined mice from the colony of Maude Slye and reached the conclusion that these cysts and neoplasms were adamantoblastomas. Since no data are available on the etiology of these spontaneous tumors, the present study seemed desirable.

Material and Methods

Sections from the heads of all mice that showed mandibular swelling at autopsy were submitted to microscopic examination during the period from August 1951 to July 1954. The animals originated from the breeding colonies or from various experimental series; most animals had been used as controls in experiments on mammary carcinoma made during this period. The animals were kept in glass cages, each containing four mice of the same strain and were given commercial food pellets and water *ad libitum*.

The head was fixed for 2 days in susa solution and the skin was dissected off. Decalcification in Da Castro's mixture [Lendrum (*2*)] was continued for 2½ weeks. Five frontal slices were then made of the part of the head between the ear and the anterior edge of the lower incisors and embedded in paraffin after 2 days in methyl benzoate celloidin. From each block as many sections as necessary were cut for examination; serial sections were made on several occasions. In some cases horizontal or sagittal sections were examined.

Animals of various strains and various age groups, which by gross appearance were normal, were used as controls—the heads being examined in the same manner. The total number of heads studied was about 700.

[1] Received for publication June 24, 1955.

Observations and Results

Anatomy

The cysts and neoplasms were found in the alveolar sockets of the lower incisors.

Both halves of the mandible and both maxillae of mice carry a single large incisor, homologous to I_1 [Addison and Appleton (*3*)], and three molars. The incisors are curved, the curvature of the upper incisors being more marked than that of the lower. The mandibular incisors extend backward, past the molars.

The teeth of the mice show continuous growth throughout their lifetime. New dentine is continuously formed at the base of the teeth, pushing them outward, but, as a result of gnawing, there is a steady wearing-off process at the outer ends. The convex side of the incisors

TEXT-FIGURE 1.—*Mandible of a mouse.* Long incisor tooth lying in wide, long alveolar socket. Dorsal surface attached with fibers to mandible and ventral surface covered with enamel formed by prismatic ameloblastic epithelium. In anterior end of socket epithelium merges into gingival stratified epithelium. Below tooth, socket is spacious and filled with loose connective tissue (*x*). Here cysts and tumors may occur.

is lined with a layer of enamel that is formed by epithelium which covers the ventral side of the lower incisors. This epithelium extends from the base of the tooth to the gingiva and shows several layers; the basal cells are flat or cuboidal and closely connected with the capillaries; and the cells nearest to the tooth are high and prismatic or cylindrical (ameloblasts). Outside the alveolar socket, no cylindrical cells are found. The epithelium, then, consists of a few layers of flat cells and merges into the pavement-cell epithelium of the gingiva. On the concave side, the tooth is firmly connected with the mandibular bone by fibers. On the ventral and the lateral sides, the alveolus is large and filled with loosely meshed connective tissue containing wide blood vessels. Cysts and tumors were found in this periodontal connective tissue (text-fig. 1).

Cysts

In the course of this investigation, cysts were found in 118 animals, in which a swelling of one of the branches of the mandible was demonstrable *in vivo* or at autopsy.

The cysts contained fluid that was clear in some cases and purulent or hemorrhagic in others. Microscopic examination revealed that the walls of the cysts were lined with epithelium. This epithelium was flat, sometimes comprising a few layers, and resembled flat pavement-cell epithelium (fig. 4). The cysts were nearly always monolocular. Examination of serial sections showed that the epithelium of these cysts was continuous with the enamel-forming epithelium—although, as a rule, only over a limited surface (fig. 5). The cysts contained desquamated epithelial cells, sometimes numerous leukocytes, and sometimes an abundance of erythrocytes. The alveolar socket was markedly enlarged (as if "inflated") by these cysts. In several cases this caused a displacement of the tooth, as a result of which it no longer fitted tightly against the corresponding upper incisor. These teeth were, therefore, no longer ground by the corresponding incisor during gnawing and did not undergo the normal wearing-down process. Growth was frequently observed to continue until the tooth touched the upper lip or the palate. At this point the mouth could not be closed and the animal was no longer able to eat and died.

A number of inflammatory cells were usually found in the periodontal connective tissue surrounding the cyst, and abscesses were sometimes formed. Abscesses may also originate from pus-filled cysts, the epithelium of which has largely disappeared. Eruption of these abscesses is possible.

Surprisingly, hairs were found in the cyst or in the surrounding tissue in *all* animals with cysts (fig. 4). The hairs, localized in the connective tissue, were surrounded by an inflammatory reaction, often associated with the formation of giant cells. That these structures were indeed hairs was ascertained with polarization microscopy and with histologic stains that stained them exactly in the same way as the hairs of the skin.[2] The significance of these hairs will be discussed later. Some cysts contained a few food particles.

Examination of the sections showed that microscopic changes often existed in the apparently normal contralateral half of the mandible, *e.g.*, the appearance of microscopic cysts (hereafter called microcysts), inflammatory processes, and hairs. Investigations were therefore extended to normal animals (see: Control Investigations).

The grossly visible epithelial cysts (hereafter called macrocysts) were found in animals 11 to 29 months of age (table 1). Most of these animals were (O20 $\times$ DBAf)F_1 hybrids or backcrosses of these hybrids with DBAf (table 2). Sixty to 70 percent of the animals autopsied after 14 months of age were hybrids of strains O20 and DBA. Of the animals with an intramandibular macrocyst, 88 percent (104 of 118) were hybrids

[2] A good method for staining hairs is Ziehl-Neelsen's method.

TABLE 1.—*Age distribution of 118 intramandibular macrocysts*

Age in months	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Number of animals	1	0	3	2	2	1	7	13	7	12	22	16	13	10	4	1	1	2	1

TABLE 2.—*Strain and sex of 118 mice with intramandibular macrocysts*

Strain and hybrid	Number (♂)	Number (♀)	Total	Average age (*months*)
(O20 × DBA or DBAf) F_1*	10	51	61	22
[(O20 × DBAf) × DBA or DBAf]bc	13	30	43	21
DBA and DBAf	1	6	7	18
RIET	1	0	1	(11)
Q	0	1	1	(21)
WLLf	0	1	1	(17)
C3Hf	0	4	4	20
Total	25	93	118	

*In hybrids the maternal parent is designated first.

of these strains. Therefore, some disposition for cyst formation among these animals must exist.

Carcinoma

Carcinoma was found in the alveolar sockets of 59 animals showing mandibular swelling.[3] These tumors were of the epidermoid type; some contained differentiated parts showing keratinization while other tumors showed anaplastic areas with spindle-shaped cells and abundant mitoses (figs. 6 and 7). As a result, parts of the tumors resembled sarcoma. The tumors showed infiltrative growth and invaded the mandibular bone. Some penetrated the bone completely and infiltrated the soft parts. The mandible was expanded by the tumors and greatly deformed. In several cases a marked osteoplastic reaction resulted. The tooth was displaced and showed malformation. The growth of these tumors was therefore undoubtedly malignant.

In order to determine whether the behavior was malignant in other respects, too, the lungs of 6 animals with these tumors were submitted to microscopic examination. Small pulmonary metastases were, in fact, found in 3 mice (fig. 8). In 2 animals metastasis was found in a cervical lymph node. Transplantation experiments confirmed the malignancy of these tumors. Tissue from 5 tumors was grafted subcutaneously and of these 4 took, grew progressively, and could be maintained during many transfer passages. Histologically, these grafted tumors resembled the original tumor, but in 1 case the cells became spindle-shaped and sarcomatoid after 5 passages.

The carcinomas were found in animals 14 to 28 months of age (table 3), of which 49 belonged to, or were closely related to, strain O20 (table 4). Only 10 animals were not related to strain O20. The fact that 83 percent

[3] In a few animals we have observed other primary tumors in the mandible: 5 osteofibromas, 1 fibrosarcoma, 1 adenocarcinoma, and 1 hemangioma. These tumors will not be discussed in this article.

TABLE 3.—*Age distribution of 59 animals with intramandibular carcinomas*

Age in months	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Number of cases	2	1	2	5	4	5	8	7	9	8	5	1	0	1	1

TABLE 4.—*Strain and sex of 59 animals with intramandibular carcinoma*

Strain and hybrid	Number (♂)	Number (♀)	Total	Average tumor age (*months*)
O20	1	9	10	19
(O20 × DBA or DBAf)F_1	5	17	22	22
[(O20 × DBAf) × DBA or DBAf]bc	5	12	17	20
WLL and WLLf	4	1	5	19
C3Hf	2	2	4	21
Af	0	1	1	(21)
Total	17	42	59	

of the animals showing intramandibular carcinoma were related to strain O20 emphasizes the significance of this strain in these investigations. The importance of this strain in investigations on mammary cancer has been stressed in a previous report (*4*).

Microscopic examination of the carcinomas revealed two special features. Nearly all of them showed a cavity or crevice corresponding to the above-described cysts (fig. 3). In some cases, the cyst was still largely intact; only part of the wall showed carcinomatous epithelium with invasive growth into the connective tissue of the alveolus. In other cases, carcinomatous epithelium was found at various points in the cyst wall. Some carcinomas showed large cavities and others, flat clefts as remnants of the cysts. These findings indicate that the carcinomas developed from the cysts (figs. 9 and 10). The flat epithelium of the cysts is, therefore, the origin of these intramandibular epidermoid carcinomas.

The second feature of importance was the presence of *hairs* in all carcinomas. The hairs were sometimes sparse but in several cases abundant (figs. 10 and 11). In the description of the cysts, it has been mentioned that hairs were found in the alveolus in all cases of cyst. Therefore, since the carcinomas arise from the epithelium of the cysts, it was to be expected that hairs would also be found in the carcinomatous tissue. The significance of these hairs will be discussed in detail.

Like the animals with cysts, those showing carcinoma often revealed lesions in the seemingly unaffected contralateral half of the mandible. These lesions included inflammatory processes and microcysts, while hairs were again found in the tissue.

Control Investigations

In view of the fact that lesions were frequently found in the presumably unaffected half of the mandible in animals examined for mandibular swelling caused by a cyst or a tumor, it was regarded as desirable to extend investigations to the mandibles of apparently normal animals.

The heads of 446 mice of various strains and age groups with externally normal teeth and mandibles were submitted to microscopic examination in the manner previously described (tables 5 and 6). Hairs were found in the alveoli of numerous animals, either unilaterally (127) or bilaterally (233). The hairs were localized along the longitudinal direction of the alveolus in the connective tissue laterally underneath the incisor (fig. 12). They were surrounded by giant cells and inflammatory infiltrates. The hairs were particularly numerous in the anterior part of the alveolus, but in some cases they extended throughout the alveolus as far as the root of the incisor. On the anterior side of the alveolus, the hairs penetrated the gingival epithelium, often splitting the anterior part of the enamel-forming epithelium (fig. 13). This splitting process resulted in the formation, in numerous cases, of what may be called an epithelial sac (fig. 14).

In 162 of the 446 control animals, small cysts were found in the mandible (fig. 15), either unilaterally (100) or bilaterally (62). The structure of

TABLE 5.—*Occurrence of hairs and microcysts in the alveolar sockets of 401 control mice 14 months of age or older*

Strain and hybrid	Number of animals	Intramandibular hairs			Intramandibular microcysts		
		None	Unilateral	Bilateral	None	Unilateral	Bilateral
O20	39	1	4	34	13	11	15
(O20 × DBAf)F_1	43	3	14	26	16	15	12
DBA and DBAf	54	7	15	32	22	20	12
CBA	74	10	25	39	63	8	3
C57BL	30	10	9	11	24	5	1
A and Af	30	13	11	6	27	2	1
WLLf	19	0	0	19	4	5	10
C3Hf	112	21	38	53	76	29	7
Total	401	65	116	220	245	95	61

TABLE 6.—*Occurrence of intramandibular hairs and cysts in young control mice*

Strain and hybrid	Number of animals	Age (*months*)	Intramandibular hairs			Intramandibular cysts		
			None	Unilateral	Bilateral	None	Unilateral	Bilateral
O20	7	10–12	3	1	3	4	2	1
(O20 × DBAf)F_1	7	6–14	3	3	1	5	2	0
DBA and DBAf	5	6–10	1	0	4	4	1	0
CBA	6	8–12	2	2	2	6	0	0
C57BL	5	8–13	1	2	2	5	0	0
A and Af	5	7–11	2	2	1	5	0	0
Total	35	6–14	12	10	13	29	5	1
(O20 × DBAf)F_1	10	1–2	9	1	0	10	0	0

these microcysts corresponded in detail to that of the macrocysts described. The occurrence of mandibular cysts in mice is, therefore, by no means rare. As a rule, however, the cysts are so small that no external swelling of the mandible is seen (microcysts). Only in some cases did the cyst grow to such an extent that the mandible contained a macroscopically observable tumor (macrocysts).

Of the 892 alveoli of lower incisors, there were 299 without hairs. No cysts were found in the latter alveoli. Hairs were found in 593 alveoli, 224 of which also contained cysts. In *all* cases in which microcysts were found in control animals, the alveolus contained hairs. This warrants the conclusion that the presence of hairs in the alveolus is a required condition for the occurrence of cysts. The presence of hairs along the upper incisors has been observed but is a much rarer finding. Only in a few cases have hairs been found in the socket of a superior incisor, also with cleavage of the enamel-forming epithelium. In one of these cases a cyst had developed in the maxillar socket.

Hairs and Cysts

Strain differences.—Comparison of animals of various strains revealed differences in the incidence of hairs and microcysts in the mandible. Table 5 shows that old animals of strains O20, DBA, WLLf, and C3Hf have a high incidence of hairs in the mandible, as have the (O20 × DBA)F_1 hybrids. Hairs are less frequently found in strain C57BL, and only exceptionally in strain A.

The incidence of mandibular microcysts is, in most strains, in accordance with these findings, with the exception of strain CBA. Microcysts are very frequent in strains O20 and DBA, in the F_1 hybrids of these strains, and in strain WLLf. Strain C3Hf holds an intermediate position. Microcysts are fairly rare in strains C57BL and A. Cyst formation is also exceptional in CBA—a strain in which hairs are frequently found in the mandible.

Hairs and Age

Hairs are less frequently found in the alveoli of young animals than in older animals. Among 35 mice about 10 months of age, there were 12 without hairs in the mandible, 10 had hairs in 1 alveolus, and 13 had hairs in both alveoli (table 6). Hairs are hardly ever found in very young animals (1 to 2 months). Among 10 (O20 × DBAf)F_1 hybrids, 4 to 8 weeks of age, there was only 1 in which a few hairs were found in the anterior part of 1 alveolus. Absence of hairs from the mandibular alveoli is exceptional in these hybrids when they are older (table 5). These findings seem to suggest that hairs enter the alveoli while the animals are aging.

Sex

A difference was noted in the frequency of hairs in males and females; hairs were found bilaterally in 70 percent of the older males but only in 48 percent of the older females. The same holds true for the microcysts, which were found bilaterally in 27 percent of the males and in 9 percent

of the females. Unilateral cysts were of equal frequency in both sexes. No cysts were found in 46 percent of the males and 68 percent of the females. A total of 103 microcysts was found among 126 males and 114 among 275 females. This comparison is not quite accurate, since the male:female ratio was not exactly the same in all the groups examined.

Table 7 shows, however, that this sex-linked difference is extremely marked in strains C57BL, C3Hf, and DBAf and in (O20 × DBAf)F_1 hybrids. A corresponding difference is seen in CBA. There is hardly any difference in strains O20 and WLLf. In these strains, however, the average age of the examined males was lower (18 months) than that of the examined females (22 months). In the A strain, the number of males examined was too small to be significant. Moreover, hairs, and particularly cysts, are less frequently found in this strain than in the other strains. In general, hairs and cysts are more frequently found in males than in females.

TABLE 7.—*Occurrence of intramandibular hairs and microcysts in male and female control mice 14 months or older*

Strain and hybrid	Sex	Number	Intramandibular hairs			Intramandibular cysts		
			None	Unilateral	Bilateral	None	Unilateral	Bilateral
O20	♂	12	0	2	10	6	0	6
	♀	27	1	2	24	7	11	9
(O20 × DBAf)F_1	♂	15	0	3	12	1	7	7
	♀	28	3	11	14	15	8	5
DBA and DBAf	♂	18	1	3	14	5	4	9
	♀	36	6	12	18	17	16	3
CBA	♂	28	2	11	15	21	4	3
	♀	46	8	14	24	42	4	0
C57BL	♂	12	3	2	7	6	5	1
	♀	18	7	7	4	18	0	0
A and Af	♂	6	4	1	1	6	0	0
	♀	24	9	10	5	21	2	1
C3Hf	♂	29	1	5	23	12	12	5
	♀	83	20	30	33	64	17	2
WLLf	♂	6	—	—	6	1	1	4
	♀	13	—	—	13	3	4	6
Total	♂	126	11	27	88	58	33	35
	♀	275	54	89	132	187	62	26

Origin of Hairs

What might be the origin of the hairs found in the mandibular socket? At first we thought that these hairs might have grown from teratomatous epithelium in the cysts. However, no real hair follicles were demonstrated. The hairs appeared to be more numerous in the anterior than

in the posterior part of the alveolus, and the epithelium was split by the hairs at the borderline between gingival and enamel-forming epithelium (figs. 13 and 14).

This suggested that the hairs came from outside, perforated the epithelium, and thus invaded the alveolus. Macroscopic examination confirmed this. When one looks into the mouth of an older mouse, a few hairs are often seen lying against one or both lower incisors; the ends of these hairs have penetrated between the tooth and the gingiva. The hairs in some cases are seen in regular tufts (fig. 16). When the animals are licking and gnawing at their own pelts or those of other mice, hairs may penetrate the alveolus, whereupon they are loosened from the skin or broken off. These hairs are pushed farther into the alveolus during eating or gnawing and may thus penetrate as far as the end of the alveolus (text-fig. 2). The presence of food particles in some of the cysts and clefts in the neighborhood of the hairs is in accordance with the exogenous origin of the hairs.

That cage mates may be the source of penetrating hairs was indicated when pigmented hairs were found in the alveolar socket of an O20 white mouse which had shared a cage with strain IF dark mice.

In order to determine whether the hairs penetrating the alveolus are predominantly from cage mates or mainly the animal's own, the following experiment was made: 44 black mice, (O20×DBAf)F_1 hybrids, were each placed in a separate cage at the age of 4 weeks. These hybrids were selected because hairs and cysts frequently had been found in the mandibles of old animals of this genetic constitution. Twenty-eight of them were sacrificed at varying ages. The remaining 16 animals were each given 3 cage mates at the age of 11 months, the mates being of the Af strain in 9 cases and of the O20 strain in 7. The results are presented in tables 8 and 9.

TEXT-FIGURE 2 (*cf.* text-fig. 1).—*Development of a cyst.* A hair penetrates from outside the alveolar socket. It perforates the gingival epithelium and cleaves the enamel-forming epithelium. The latter is damaged and its cells become flat. Fluid accumulates in the cleft, which becomes cystic. The cleft is lined with flat stratified epithelium which may be the point of origin of intramandibular carcinoma.

TABLE 8.—*Intramandibular hairs and cysts in 28 (O20 × DBAf)F₁ hybrids kept in separate cages (14 ♂ and 14 ♀)*

Age (*months*)	Total number	Number with hairs in mandible	Number with cysts in mandible
5	4	0	0
7	4	0	0
11	3	0	0
14	2	0	0
17	2	0	0
19	2	0	0
22–26	11	1 (unilateral) 3 (bilateral)	1 (bilateral)

TABLE 9.—*Intramandibular hairs and cysts in 16 (O20 × DBAf)F₁ hybrids (8 ♂ and 8 ♀) caged individually until 11 months of age, and then with 3 cage mates*

Age (*months*)	Total number	Number with hairs in mandible			Number with intramandibular cysts
		None	Unilateral	Bilateral	
14	12	5	6	1	0
16–19	2	1	1	0	0
22–24	2	0	1	1	0

Hairs were not found in the alveoli of the 17 isolated animals that were killed before the age of 20 months. Of the 14 animals that had cage mates for a few months and had then been killed before the age of 20 months, 8 showed hairs in the alveoli. These hairs were not pigmented. Our findings suggest that hairs are more likely to penetrate the alveoli in the presence of cage mates and that the hairs originate from the cage mates. Eleven animals were kept in separate cages and remained alive until they showed signs of senility (22 to 26 months). Four of these animals showed bilateral occurrence of hairs in the alveoli, indicating that the animal's own hairs may also penetrate the alveoli, but this seems to take place only when the animals are very old. The majority of hairs found in the mandibular alveoli in mice originate from cage mates.

Penetration of Hairs

A summary of the factors on which penetration of hairs into the alveolus is dependent should mention environment (absence or presence of cage mates) as the first factor. Sex is a second factor; penetration is more frequently seen in males than in females (table 7). A third factor is the marked difference of penetration in the various strains (table 5). Nearly all old mice of strains O20 and DBAf and their F_1 hybrids, WLLf, CBA, and C3H have hairs in their alveolar sockets, while hairs were found less frequently in mice of strain C57BL and particularly of strain A. Further, in the former strains not only was the number of animals with hairs in the alveolus larger but also the number of hairs within each alveolus. Since the latter factor cannot be used in comparison without considerable

difficulty, only the presence or absence of hairs in the alveoli is taken into account.

The question was raised whether these strain differences may be based on hereditary differences in the anatomical structure of the mandible. A search for such anatomical differences revealed considerable variability in the structure of the enamel-forming epithelium. This epithelium has several layers, with a high cylindrical upper layer adjacent to the enamel (figs. 17 and 18). In young animals, it extends from the posterior end of the incisor far forward—nearly to the point at which the tooth emerges from the alveolus. The posterior half of the epithelium consists of high prismatic cells (fig. 17) and the anterior part, of cylindrical cells (fig. 18). At about the level where the tooth emerges from the socket, the epithelial cells become flat (fig. 19) and abruptly merge into pavement-cell epithelium, which bends and continues into the gingival epithelium (*cf.* text-fig. 1). The same condition may be found in older animals. In some, however, the epithelium is cylindrical over a smaller distance, while the flat epithelium extends underneath the entire anterior half of the tooth.

The borderline between cylindrical and flat epithelium has therefore been pushed back out of place in these cases; it may be localized underneath the molars or farther back. Particularly in animals with hairs in the alveolus, the major portion of the enamel-forming epithelium was often flat (metaplasia).

The question was raised whether this metaplasia resulted from epithelial damage caused by invading hairs or constituted a senile change which augmented the penetration of hairs. In order to settle this point, the following investigations were made: In a number of animals, serial sections were made in order to determine the borderline between flat and cylindrical epithelium in both alveoli, the presence of hairs in the alveoli, and the extent to which these hairs had penetrated the alveoli. The borderline between high and flat epithelium was invariably found in the extreme frontal part of the alveolus in animals 7 to 11 months of age of strains A, O20, and CBA and the (O20 $\times$ DBAf)F_1 hybrids (table 10). In strains DBA and C57BL, however, the borderline was found farther back, as a rule underneath the first or second molar. In older animals the borderline was often found in the posterior part and, in some animals, this was the case even when no hairs had penetrated (table 11). The displacement of the borderline, *i.e.*, the progress of the metaplasia of the cylindrical epithelium, would therefore seem to be a phenomenon of aging. This is inconsiderable in strain A, but extremely marked in animals older than 19 months of strain CBA and in (O20 $\times$ DBA)F_1 hybrids. In strain C57BL the borderline was found to be farther back than in any other strain, as was to be expected on the basis of observations made in young animals. The degree of metaplasia (of this senile type) is therefore dependent on the genetic constitution. The tendency to metaplasia is strongest in strain C57BL, followed by strains CBA, O20, and DBA with F_1 hybrids in this order. The tendency is least marked in strain A. This sequence differs from that seen when these strains are arranged

according to frequency of penetration of hairs into the alveolus [O20, (O20 × DBA)F_1, DBA, CBA, C57BL, and A]. These show that the frequency of penetration of the hairs is not dependent on metaplasia of the enamel epithelium.

In some cases, in which only a few hairs had penetrated, the epithelium directly adjacent to these hairs had become flat, while the epithelium next to it was still high and cylindrical (fig. 20). The metaplasia of the enamel-

TABLE 10.—*Borderline between cylindrical ameloblastic epithelium and stratified epithelium in mice of different strains, 7 to 11 months of age*

Strain and hybrid	Number of alveolar sockets examined	Presence of intramandibular hairs	Location of borderline				
			Anterior part	M_1*	M_2*	M_3*	Posterior part
A and Af	4	−	4	—	—	—	—
	4	+	4	—	—	—	—
O20	1	—	1	—	—	—	—
	5	+	5	—	—	—	—
(O20 × DBAf)F_1 individually caged	12	−	12	—	—	—	—
CBA	4	−	4	—	—	—	—
	2	+	2	—	—	—	—
DBA and DBAf	2	−	—	2	—	—	—
	4	+	—	2	—	2	—
C57BL	2	−	—	1	—	1	—
	4	+	—	1	2	—	1

*M = molar.

TABLE 11.—*Borderline between cylindrical ameloblastic epithelium and flat stratified epithelium in mice 16 months of age and over*

I. *Without hairs* in the alveolar socket

Strain	Number of alveolar sockets examined	Location of borderline				
		Anterior part	M_1*	M_2*	M_3*	Posterior part
A	16	10	3	2	1	0
O20	3	1	2	0	0	0
(O20 × DBAf)F_1 17–19 mo	8	8	0	0	0	0
(O20 × DBAf)F_1 22–26 mo	12	5	2	2	3	0
DBAf	3	1	0	2	0	0
CBA	8	1	2	3	0	2
C57BL	10	0	1	6	3	0

II. *With hairs* in the alveolar socket

A	6	4	1	1	0	0
O20	13	2	1	2	0	8
DBAf	12	5	2	4	0	1
CBA	8	0	1	2	0	5
C57BL	8	0	0	5	1	2

*M = molar.

forming epithelium must therefore be considered as a senile change which is augmented by the traumatic damage resulting from penetration of hairs into the alveolus; it is not the cause of the penetration of hairs.

No other anatomical differences were found between animals of strains with a high incidence of hairs in the mandibular alveoli and those in which this is rare.

Type of Hair

The question was raised whether the type of hair influenced the frequency of penetration into the alveolus. The animals examined as a rule were kept in a cage with other animals of the same strain. The fact that penetration of hair is considerably less frequent in strain A than in strain O20 might be based on the fact that hair of strain A animals penetrates less readily than hair of the O20 strain. In order to investigate this possibility, the following experiment was made: Sixteen (O20 × DBAf)F_1 animals were kept in isolation in separated small cages. At the age of 11 months, 9 of the mice were each given 3 strain A cage mates, while 7 were each given 3 strain O20 cage mates. Table 12 shows that the hairs of both strain A and strain O20 mice had penetrated the alveoli of the F_1 animals after a few months.

TABLE 12.—*Occurrence of hairs in mandibles of (O20 × DBAf)F_1 mice kept in individual cages for 11 months and then given three cage mates apiece*

Number of (O20 × DBAf) F_1 mice	Age at death (*months*)	Cage mates	Number of animals with hairs in mandible		
			None	Unilateral	Bilateral
6	14	Af	1	4	1
3	19–24	Af	0	2	1
6	14	O20	4	2	—
1	16	O20	1	—	—

There is, therefore, nothing to suggest that differences in hair type are responsible for the fact that the incidence of hairs in strain A is so much lower than in strain O20.

Behavior

The differences in extent of penetration into the alveoli in the different strains *cannot* be explained by anatomical differences; nor can it be that differences in type of hair are of importance in this respect. The most plausible explanation may be found in presumable differences in gnawing habits. Animals of the O20 strain are active gnawers and several of them are whisker-eaters, often to the extent of gnawing the heads of their cage mates completely bald. Hauschka (*5*) has found that the incidence of whisker eating increases significantly with age, which is in accordance with our observations (tables 5 and 6). The differences between males and females, too, may be attributable to more aggressive behavior on the part of the males.

Relationship between Hairs and Cysts

The fact that mandibular cysts are found only in association with hairs in the alveoli led us to the conclusion that the presence of these hairs is a required condition for the occurrence of cysts. Since it has been demonstrated that the hairs penetrate the mandible from outside, the occurrence of the cysts may be explained as follows: Hairs, when penetrating the alveoli of the lower incisors, perforate the gingival epithelium, split the enamel-forming epithelium lining the lower side of the incisor, and invade the connective tissue via these clefts (text-fig. 2). This is clearly visible microscopically. The multilayered cylindrical epithelium changes as a result of this splitting—the cells are flattened and resemble pavement-cell epithelium. The hairs, surrounded by fluid, are localized in the cleft. When a considerable quantity of fluid is formed, the crevice widens and a variety of transitional stages between cleft and cyst then occur. The cysts, therefore, should be regarded as distended crevices resulting from trauma in the enamel-forming epithelium. This explains why the cysts are invariably associated with the enamel-forming epithelium, as shown in serial sections. Why cystic distension of the cleft occurs in certain cases following splitting of the epithelium has not so far been explained. There may be some correlation with the possibility for escape of fluid from the crevices.

As a rule, these cysts are not found in the anterior part of the alveolar socket, probably because fluid from the epithelial crevices finds no obstruction here but can escape to the oral cavity. Crevices localized farther back are more likely to become cystic. Other factors, however, must be involved. In the CBA strain, penetration of hairs is nearly as frequent as in (O20 $\times$ DBAf)F_1 hybrids; the incidence of cysts, however, is considerably lower (table 5). Microcysts are extremely frequent in O20 mice, but in no case did the cysts become so large as to be noticeable externally, as was the case in the DBA animals and in (O20 $\times$ DBAf)F_1 hybrids (table 2). There are also, therefore, genetically determined factors involved in the formation and development of these cysts.

Pathogenesis of Carcinomas

The clefts and cystic remnants found in the carcinomas showed that the neoplasms arise from the epithelium of cysts. Moreover, a few cysts were found which showed the beginning of carcinomatous epithelial growth in the wall. Penetration of hairs into the alveoli is a required condition for the occurrence of cysts. From our observations, hairs were found in the alveoli in all 59 cases in which carcinoma was found.

In order to gain some impression of the frequency of the occurrence of carcinomas in cysts, the following calculations were made: The two youngest animals showing intramandibular carcinoma were 14 months old. The number of animals which were sacrificed or which succumbed at the age of 14 months or older during a period of 18 months at the laboratory was determined (table 13).

During the 18-month period, 614 mice of the O20 strain died. Carcinoma was found in 4 of these. On the basis of table 7 (taking sex differences into account) it was estimated that these 614 animals had a total of about 640 microcysts. The frequency of carcinogenesis from cysts in O20 animals older than 14 months may therefore be expressed as 4/640.

Of the (O20×DBAf)F_1 hybrids, 2,031 died during the same period. On the basis of table 7, the number of microcysts in this group was estimated at about 1,800. Thirteen carcinomas were found during this period. The frequency of carcinogenesis from cysts in these animals is therefore 13/1,800 (above the age of 14 months). This proportion hardly differs from that in the O20 strain.

The total number of animals that died after 14 months of age in the strains DBA, CBA, C57BL, and A was 1,993, with an estimated total of 760 cysts. No intramandibular carcinoma was found in any of these strains during this period. The estimated number of cysts in these

TABLE 13.—*Estimated number of microcysts and number of observed carcinomas in animals which died at 14 months of age or older during a 1½-year period*

Strain	Number of animals	Sex		Estimated number of microcysts	Number of animals with observed carcinoma
		(♂)	(♀)		
O20	614	139	475	640	4
(O20×DBAf)F_1	2,031	637	1,394	1,800	13
DBA and DBAf	517	244	273	450	0
CBA	532	230	302	110	0
C57BL	543	320	223	150	0
A and Af	401	143	258	50	0

animals, however, was so high that, if the frequency of carcinogenesis from the cysts were as high as in O20 and (O20 × DBAf)F_1 animals, 5 or 6 carcinomas could have been expected.

During the year following the end of the period to which table 13 applies, several carcinomas were encountered—4 in the O20 strain and 9 among (O20 × DBAf)F_1 hybrids. Again, no carcinomas were found in CBA, DBA, and C57BL animals. However, a carcinoma of a cyst wall was found in a 21-month-old Af female. This shows that intramandibular carcinoma may well occur in animals not related to the O20 strain (as shown also by the cases in strains WLL and C3H in table 4). There is, therefore, a significant difference between strain O20 and (O20 × DBAf)F_1, as opposed to strains DBA, CBA, and C57BL with respect to the chance of development of carcinoma in intramandibular cysts.

Possible correlations between this difference and the age at death of the animals were investigated. The average age of the A and DBA animals was slightly lower than that in O20. The average age in strain CBA was higher and that in C57BL about equal to O20. The (O20×DBAf)F_1 hybrids had a higher average age (table 14). The

TABLE 14.—*Age and number of animals of different strains which died during a period of 1½ years*

Strain	Number of animals	Age at death (*months*)			
		14–18	18–22	22–24	Over 24
DBA and DBAf	517	264	207	41	5
CBA	532	190	166	107	69
C57BL	543	170	191	161	21
A and Af	401	180	155	52	14
O20	614	199	232	155	28
(O20 × DBAf)F_1	2, 031	554	505	487	485

average in the strains DBA, CBA, C57BL, and A practically tallied with the mortality line of O20.

As indicated by the data in table 14, differences in carcinoma frequency are not attributable to a longer lifetime in O20 animals.

If the cysts in O20 occurred at an earlier average age than in the remaining strains, this would explain the higher frequency of carcinoma in cyst walls, in the long run, in O20 animals. Investigations were therefore made in order to determine whether cysts occurred earlier in the O20 strain.

Table 15 shows that the microcyst frequency in the O20 strain is continuously on the increase until old age. In DBA, the cysts show virtually the same frequency and occur in about the same age groups. In CBA, they would seem to occur somewhat later, but cysts are of such low frequency in this strain that the number of young animals examined was too small to warrant definite conclusions regarding the occurrence of cysts among the younger age groups. However, although cysts are rare in strain A mice too, cysts in younger animals were found. The youngest C57BL animal in which a cyst was found was 21 months old; here too, however, the number of young animals is insufficient to permit conclusions to be drawn.

TABLE 15.—*Incidence of intramandibular microcysts in control animals at different ages*

Strain	8–14 months		14–18 months		18–22 months		22→months	
	Number of animals	Number of cysts	Number of animals	Number of cysts	Number of animals	Number of cysts	Number of animals	Number of cysts
O20	7	3	7	5	19	19	13	17
DBA and DBAf	4	1	7	6	21	22	26	16
CBA	6	0	12	0	9	0	53	14
C57BL	5	0	7	0	3	1	19	6
A and Af	5	1	11	0	11	3	8	1

There is no doubt that most cysts did not occur until old age in O20, while in some other strains (such as DBA) cysts may occur in young animals. The differences would seem to be too small to explain the absence of carcinoma from other strains, particularly from the DBA strain. This suggests differences among various strains in predisposition to the

development of cancer in otherwise comparable cysts—differences dependent on genetic constitution.

Discussion

Morphologic observations in mice have shown that tumors which were identified as epidermoid carcinoma occur in the mandible. These carcinomas arise from cysts formed as a result of traumatic splitting of the enamel-forming epithelium which lines the lower side of the incisors. The splitting is caused by hairs, which penetrate the alveolar socket from outside.

Similar tumors and cysts have been described in detail by Zegarelli (*1*) in 1944, in mice from the laboratory of Maude Slye (*1*). The pathogenic mechanism is not described in his report and no mention is made of the presence of hairs. One of the illustrations (No. 5), however, shows unmistakable hairs in a mandibular cyst (indicated by G-artefact).

It would seem to be beyond doubt that the tumors described by Zegarelli are identical with those under discussion; the pathogenic mechanism, therefore, is probably the same. The striking feature of this mechanism lies in the fact that traumatic epithelial damage caused by penetrating hairs is a required condition. This penetration of the mandibular sockets by hairs is very common in mice, and we have observed it a few times in rabbits, rats, and hamsters.

Penetration of the hairs is influenced by various factors, including environment (presence or absence of cage mates), sex, and genetic constitution. The influences of sex and constitution are probably based on differences in behavior and gnawing habits.

Differences in the frequency of penetration of hairs into the alveoli were not attributable to anatomical differences between the strains or to differences in properties between the various types of hair.

The epithelium, once split, degenerates and assumes a resemblance to pavement-cell epithelium. The same is observed without preceding traumatic damage in old age (as a senile phenomenon); it is more marked in some strains than in others.

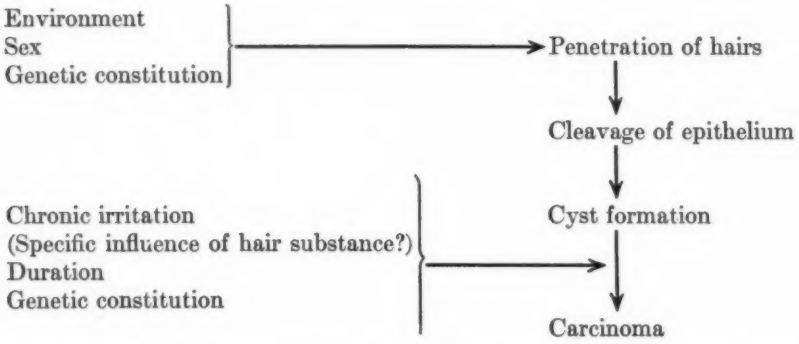
Factors causing the development of cancer in the epithelium of the cysts have been studied. Two properties of this epithelium may be of importance in this respect. First, the epithelium is not the normal enamel-forming epithelium but is subject to metaplastic changes and shows a structure reminiscent of pavement-cell epithelium. Second, the epithelium is affected by chronic inflammation of the surrounding connective tissue into which hairs have penetrated; hairs and inflammatory cells are also found in the lumina of the cysts. Such a chronically irritated metaplastic epithelium carries a higher risk of becoming subject to malignant changes—a fact known from human pathology. However, this risk is not very high—malignancy develops in less than 1 percent of the cysts!

The hair itself may be a factor of importance in this respect. Various macromolecular substances may have a carcinogenic effect [Oppenheimer

(*6*), Druckrey (*7*)] and, although this has not hitherto been demonstrated for hairs or keratin, the possibility should be taken into account. Peacock has implanted whiskers—his own—subcutaneously in mice. Granulomatous reactions followed, but no tumors resulted (*8*).

For the time being, we are inclined to regard chronic irritation and metaplasia as the primary factors in carcinogenesis in intramandibular cysts.

The tumors occur in animals with and without the mammary tumor agent and there is no demonstrable preference for either group. There was a marked preference, however, for O20 animals and related hybrids. This was partly attributable to the high frequency of cysts in these animals. However, various findings suggested the strong probability that a greater predisposition to the development of carcinoma in the epithelium of the cysts existed in the genetic constitution of strain O20 than in that of strains DBA, CBA, and C57BL. Text-figure 3 summarizes the various factors which, according to our studies, influenced the occurrence of malignant growth in the mandibles of mice.



TEXT-FIGURE 3.—Factors involved in the development of intramandibular carcinoma in mice.

Zegarelli's term "adamantoblastoma" would seem to be less suitable for these tumors and cysts, since the term suggests analogy to human adamantoblastomas. These mouse tumors, however, are of a different nature. We would suggest the term "intramandibular carcinoma."

Summary

Most old mice appear to have hairs within their mandibular incisor sockets.

These hairs are exogenous and penetrate the mandible, cleaving the gingival and enamel-forming epithelial layers. Most hairs are from cage mates, as was proved with mice kept in individual cages. The incidence of the presence of hairs in the mandible varies among the different strains. This strain difference is probably attributable to behavioral differences (gnawing habits). More hairs are found in the mandible in males than in females.

Many old mice have small cysts in their mandibular sockets; a few of these cysts grow and become large. Every mandible with a cyst also contains hairs, and all phases of transition between clefts and fully developed cysts in the enamel-forming epithelium are found. The cysts prove to be distended clefts resulting from the traumatic cleavage of the epithelium caused by penetrating hairs.

Carcinoma occurs in the alveolar socket in nearly 1 percent of the old animals related to the O20 Amsterdam strain. These tumors have their origin in the cysts. Chronic irritation of the degenerated epithelium of the inflamed cysts may be the main factor involved here in carcinogenesis.

Mice of the O20 Amsterdam strain have a greater disposition for the development of cancer in intramandibular cysts than certain other strains.

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PLATE 50

FIGURE 1.—Head of a mouse from the O20 Amsterdam strain with a tumor of the mandible.

FIGURE 2.—Frontal section through the head of a mouse with a cyst in the mandible. B=brain; E=eye; M=mouth; T=tongue. On the *right* there is a normal mandible. *Below* its incisor there is a cleft (*A*). This cleft contained enamel that was dissolved during decalcification. On the *left* side, the alveolar socket is enlarged by an intramandibular cyst (*C*). × 5

FIGURE 3.—Frontal section through the head of a mouse with an intramandibular carcinoma (*T*). In the carcinomatous tissue the remnant of a cyst (*C*) can be seen. × 5

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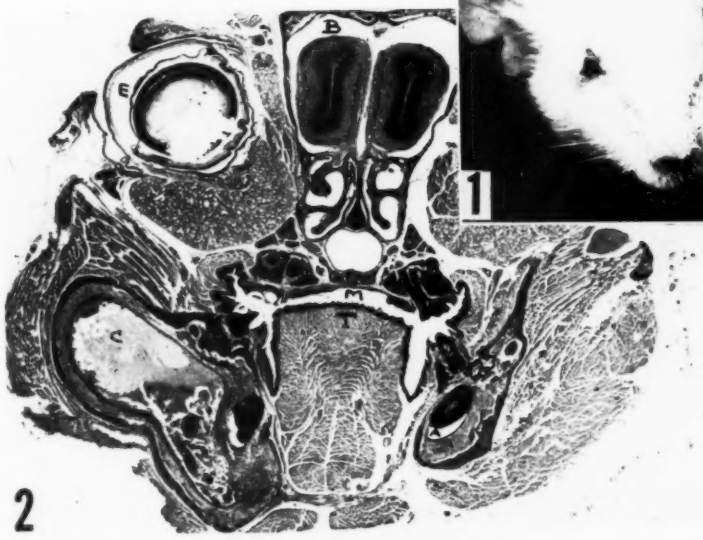

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PLATE 51

FIGURE 4.—Wall of an intramandibular cyst containing macrophages, desquamated epithelial cells, and leukocytes. The lining is stratified epithelium. Hairs can be seen in the lumen, in the epithelium, and in the surrounding connective tissue (two hairs are indicated by an *arrow*). × 160

FIGURE 5.—Horizontal section through the mandibular region. The front end is on *top*. The *right* mandible contains a small cyst (*C*); the swollen *left* mandible contains a large cyst. The epithelium of the large cyst is in this section (*between the arrows*) continuous with the enamel-forming epithelium of the incisor. × 10

FIGURE 6.—Intramandibular carcinoma of epidermoid type with keratinization and pearl formation. × 300

FIGURE 7.—Undifferentiated anaplastic intramandibular carcinoma. × 300

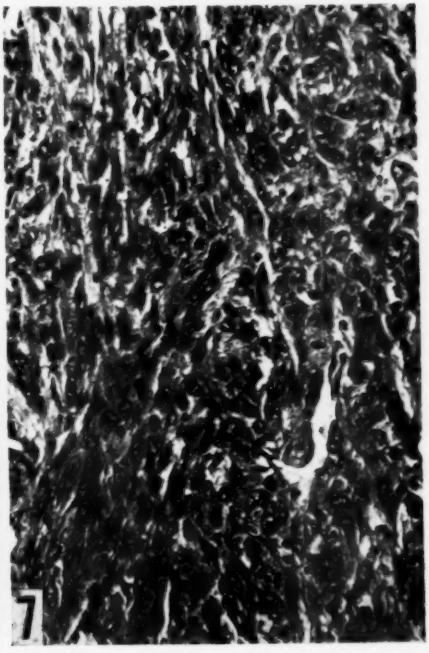

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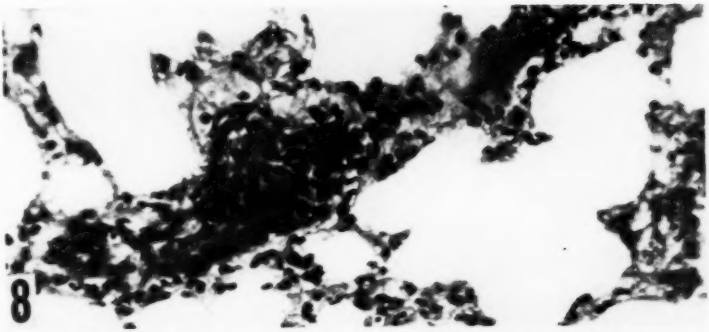
PLATE 52

FIGURE 8.—Metastasis in the lung from an intramandibular carcinoma. × 300

FIGURE 9.—Intramandibular carcinoma originating from a cyst. The lining epithelium of the cyst is pleomorphic (*right side*). The adjacent connective tissue (*below*) is invaded by strands of cancer cells. × 160

FIGURE 10.—Another carcinoma arising from the epithelium of an intramandibular cyst. Part of the lining epithelium is pleomorphic and invasive. The surrounding tissue is full of anaplastic malignant cells. Several hairs can be seen. × 160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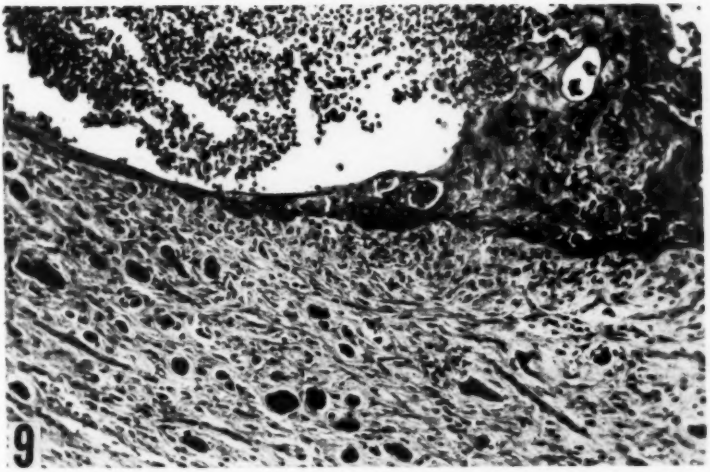
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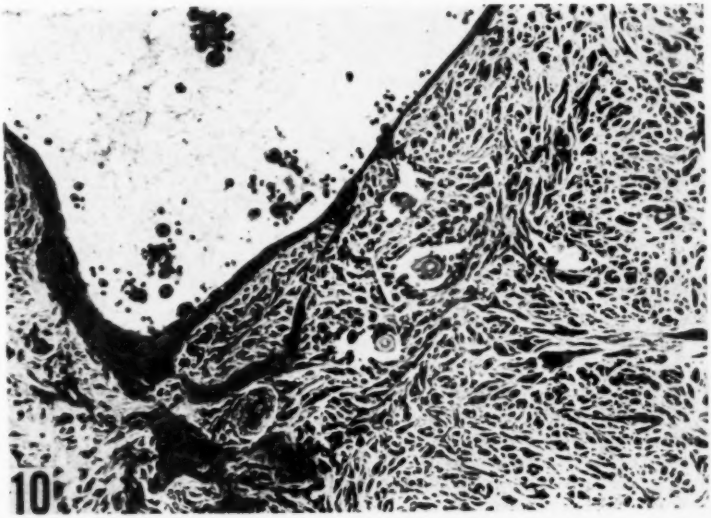
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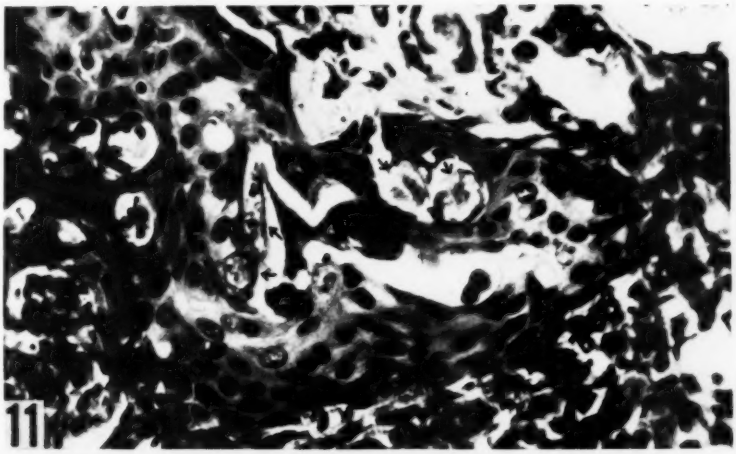
PLATE 53

FIGURE 11.—Intramandibular epidermoid carcinoma. Several cross-sectioned hairs are lying in the carcinomatous tissue (*indicated by arrows*). × 350

FIGURE 12.—Horizontal section through the mandible. Several hairs, cut longitudinally, are lying in the paradental tissue parallel with the long axis of the alveolar socket. × 180

FIGURE 13.—Sagittal section of the anterior part of the mandible. Two hairs penetrate from outside (*right side*) and cleave the frontal part of the enamel-forming epithelium. The same phenomenon is shown in frontal section on figure 14. × 160

A: Space which has been filled by enamel before it was dissolved during calcification.
C: Traumatic intra-epithelial cleft.
G: Gingival epithelium.
I: Incisor.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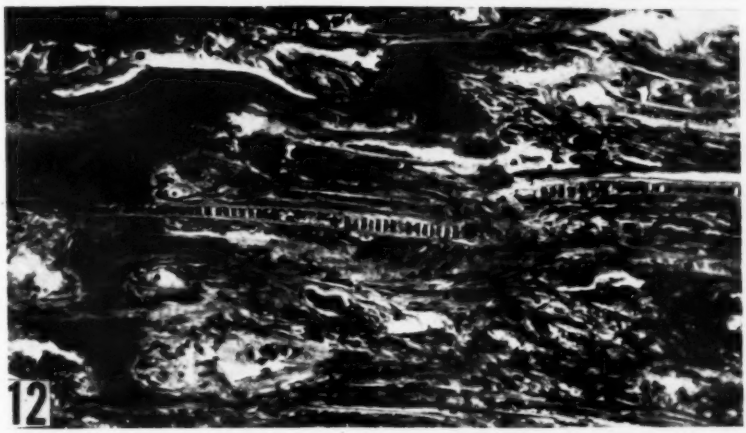
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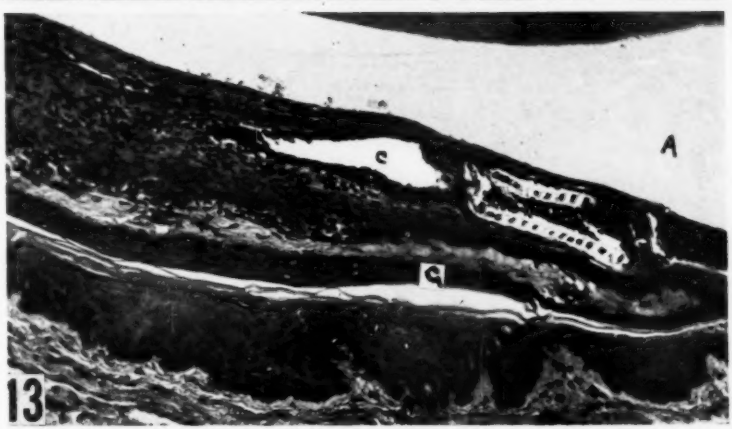
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PLATE 54

FIGURE 14.—Frontal section through the anterior part of the mandible. The enamel-forming epithelium of the left side has been cleft by penetrating hairs. The same phenomenon is shown in sagittal section on figure 13. × 20

FIGURE 15.—Frontal section through the mandibular region of a control animal of normal appearance. At the *right* there is an intramandibular microcyst (*C*). × 12

FIGURE 16.—Front view of the mouth of an (O20 × DBAf)F_1 hybrid mouse. Numerous pigmented hairs are sticking into the alveolar sockets of the lower incisor teeth.

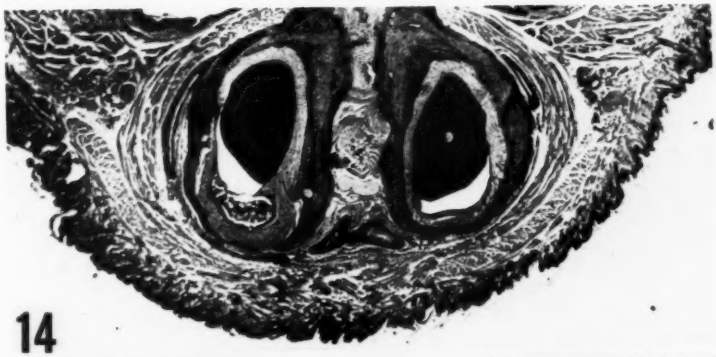

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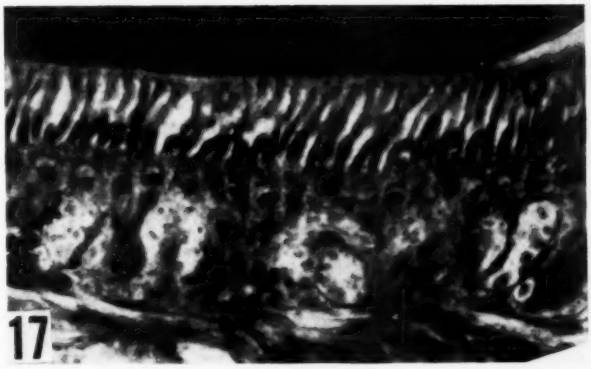
PLATE 55

FIGURE 17.—Enamel-forming epithelium with high prismatic ameloblasts (posterior part of the epithelium). × 480

FIGURE 18.—Enamel-forming epithelium with cylindrical cells (anterior part). × 480

FIGURE 19.—Flat epithelium, the transition between stratified gingival and enamel-forming epithelium. × 480

FIGURE 20.—Frontal section. Enamel-forming epithelium with cylindrical cells (*left*) and degenerated flat cells (*right*). Many hairs (*arrows*) have penetrated the subjacent stroma. × 240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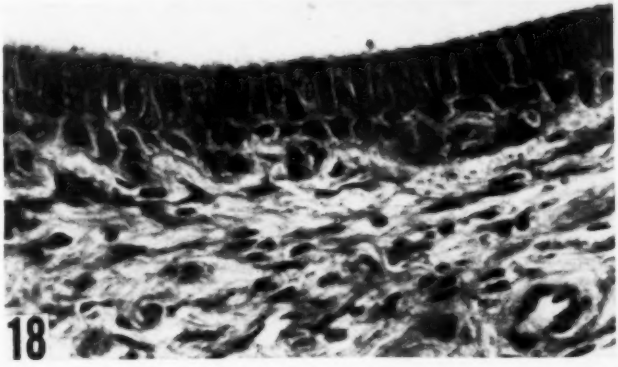
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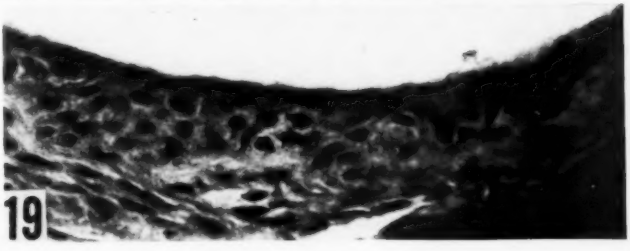
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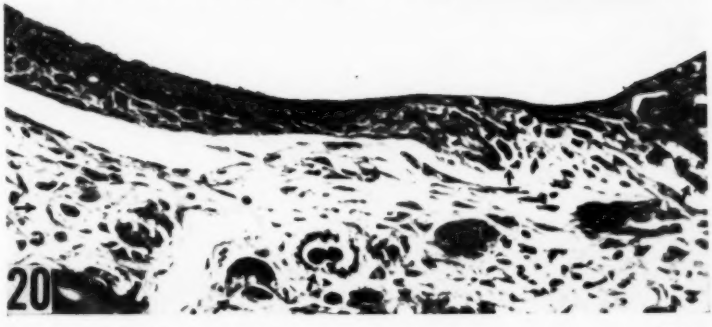
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Mammalian Chromosomes *in Vitro*. VI. Observations on Mitosis with Phase Cinematography[1,2]

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The stage that is most satisfactory for accurate analysis of mitosis in living cells is anaphase. Not only can the beginning and the end of the chromosome movements be determined rather critically, but also the time-motion relationships of the chromosomes and the spindle can be plotted. Indeed, interest has been concentrated previously on this stage, and several detailed reports are available for both plant and animal material. Bělař's (*1*) study of *Chorthippus* and Barber's work (*2*) on *Tradescantia* are among the earlier reports on this problem. Ris (*3*) distinguished two steps in the movements of chromosomes during anaphase in *Tamalia*, namely, their movement toward the poles, or the shortening of the chromosomal fibers, and the elongation of the spindle body, which further separates the chromosomes. Hughes and Swann (*4*), working with chick fibroblasts, and recently Boss (*5*), using newt fibroblasts, agree with Ris' view. Boss also states that there is a constant time relation between spindle elongation and cleavage—that is, cytoplasmic cleavage and the second anaphase movement begin simultaneously.

Recently, attention has been directed to the movements of the chromosomes before metaphase. It has been found (*6*) that endosperm is good material for studies on living cells in division; Bajer (*7*) reports the metakinetic movements in living cells of *Haemanthus katherinae*.

It is evident that phase-contrast cinematography has the advantage of accelerating the movements while permitting the correct recording of the time-motion relationships, so that events taking place in a living cell can be studied both on the screen and by single frame projections. Even so, it is not very easy to follow a cell from the onset of prophase to the interphase of its daughter cells, since we lack definitive criteria by means of which the mitotic event can be predicted. A rapidly growing culture usually provides numerous dividing cells at all stages, but in records at high magnification of a single cell there is only a very small chance that

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[2] Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council administered by C. M. Pomerat and by a grant-in-aid from the Damon Runyon Memorial Fund for Cancer Research, Inc., administered by the author.

[3] The author wishes to thank Dr. C. M. Pomerat who kindly supplied the films for the present report.

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it will divide and thereby provide a complete picture of mitosis. A compromise method consists of using lower magnifications (8-mm. objective) to cover a larger field and relatively high acceleration (one frame per minute), but under these conditions nuclear phenomena are usually obscured.

In laboratories where time-lapse cinematography is used as a routine technique, motion-picture sequences of mitotic divisions are often obtained as a by-product of various research projects. The present study represents the results observed in several selected sequences of cell division in cultures of various mammalian tissues; they were photographed at various times by workers in the Tissue Culture Laboratory of the Medical Branch, University of Texas. Some footage of abnormal mitoses was also chosen because of the rarity and the significance of such anomalies in the field of dynamic cytology.

Materials and Methods

Motion-picture sequences were selected for clarity of mitotic division from films that had been taken for various research projects. With one exception, in which a cell from a culture of cat cerebellum was traced from the beginning of prophase to the end of reconstitution, the sequences began at full metaphase.

All the cultures were set up, following the routine of this laboratory, in roller tubes with an avian plasma clot. Usually 4 or 5 explants were mounted on each of two 12 × 50 mm. cover slips which were then placed in a test tube. The following liquid medium, in 2 ml. amounts, was added to each tube and the fluid was changed twice a week: human ascitic fluid, 50 percent; Gey's balanced salt solution, 45 percent; and embryonic extract, 5 percent.

All ciné records were made with a 1 mm. dark phase-contrast oil-immersion lens (American Optical Co.) with or without the use of a 6× Leitz eyepiece. The temperature in the box containing the microscope varied between 35 to 37° C. Most of the sequences were taken at 8 frames per minute, but a few were made at 17 and one at 4 frames per minute. Since the conditions for the time lapse were recorded in every film and the frame counter also showed the exact intervals employed, accurate time analyses of the mitotic events could be made.

Each sequence was projected on a screen with silent motion-picture speed (16 frames per second) for general study and was later examined on a film-splicer viewer to record the events that had taken place. The film was then projected with the aid of a Leitz Focomat IIA enlarger to observe the details of the process. For every half minute of film time the outlines of the cell and the chromosomes were traced on paper, and measurements of anaphase movements and cleavage were later made against a micrometer scale projected onto the paper. Since the sequences were not specifically taken for the purpose of analyzing mitotic division, the focus had not been set on particular chromosomes; consequently, in most cases the focal plane varied and tracing work on any particular object was not possible. The

records for anaphase movements were therefore made by averaging the centromere locations of the chromosome groups. The error of the measurements was greater than would have resulted from marking a single pair of centromeres, but this probably did not affect the curves seriously.

The starting point for the estimation of the rate of cleavage was made by measuring the cell width at the metaphase plate. During cleavage the measurement was made across the center of the constriction where the width of the cell was narrowest. Thus, for each cell, a curve for the increase in distance between the two centromeric groups representing anaphase and a curve for the decrease in cell width representing the cytokinetic process were plotted.

Photomicrographs were made by enlarging selected frames of the film strip with the Leitz Focomat IIA enlarger on X-ray film negatives and prints were made from these.

Observations

Thirty-six sequences were analyzed, including cells from 1 rat, 1 cat, 2 monkeys (*Macaca mulatta*), and 32 human cells. Among the human cells 16 were from the HeLa strain.

With the exception of a few special cases which will be described more fully, data concerning movements of the chromosomes during anaphase and the cleavage process are summarized in table 1. Three sequences, one from monkey brain, one from human testis, and one from the HeLa strain were selected to demonstrate the curves of anaphase and cleavage movements (text-figs. 1–3). It can be seen in table 1 that considerable variation may occur even among cells of the same type in the same culture. For instance, anaphase was completed within 4 minutes in 2 of the 7 human testicular spindle cells, whereas the other 5 cells took twice as long. The pattern of anaphasic movement of the chromosomes is likewise diversified. For instance, in some cells there was a definite pause period of approximately half a minute during the early period of anaphase (text-fig. 1), whereas in others this may have been obscured or entirely absent. The late period of anaphase also shows diversity, for in some cells anaphase ended in a slow, gradual movement which sometimes produced irregularities in the curve and lasted for several minutes (text-fig. 2), but in others the chromosomes completed their travel rather abruptly.

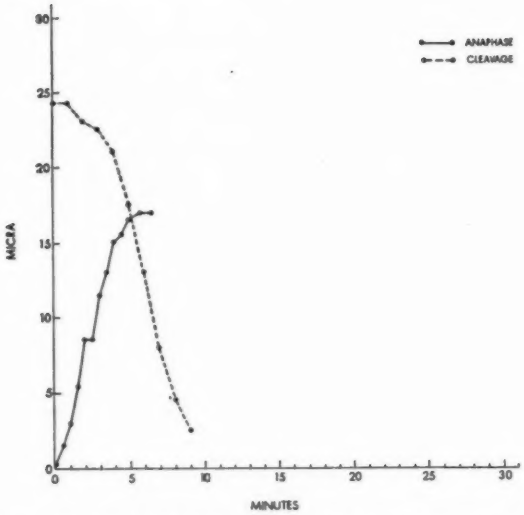
In a few cases the earliest evidence of cleavage was coincident with the beginning of the second period of anaphase, but in most cells the beginning of cleavage occurred in the late period of anaphasic movement. Most of them showed a rapid middle period with slower movement at both ends.

It is of interest to note that the average distance traveled by the chromosomes of the HeLa cells (15 μ) was shorter than that of the nonmalignant cells (19.4 μ), although the average width of the HeLa cells (24.5 μ) slightly exceeded that of the nonmalignant elements (23 μ). Also, the time required for completion of the cleavage process was definitely longer in the HeLa strain, even excluding the extremely abnormal sequence 72 IX R_2, in which the cleavage process took 61 minutes. The correlation

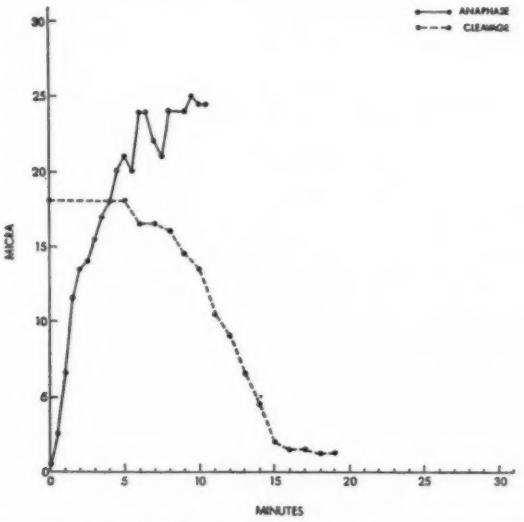
TABLE 1.—*Time-motion relations in 36 ciné sequences of anaphase and cleavage of mammalian cells in tissue culture*

Film No.	Organism	Tissue	Cell	Anaphase (*total minutes*)	Pause period after beginning of anaphase	Distance between final opposite centromeres (μ)	Width of cell at metaphase plate (μ)	Beginning of cleavage after beginning of anaphase (*minutes*)	Cleavage (*total minutes*)
57 VIII L_2	Human	Testis	Spindle	9	Obscure	21	23	4	10
57 VIII L_3	"	"	"	3½	None	17	20	2	7
57 VIII L_4	"	"	"	10	Obscure	24½	18	6	10
57 VIII L_5	"	"	"	9	1½–2	24	23	6	10
25 IX R_1	"	"	"	9	None	22	21	7	8
25 IX R_2	"	"	"	4	Obscure	17½	22½	2	5
25 IX R_4	"	"	"	8½	"	23	23½	4	7
50 IX R_4	"	Fetal spinal ganglion	"	7½	None	22½	23	5	11
46 VII R_3	"	Tenon's capsule	"	8	2–2½	26	28	7	10
78 VII R_3	"	Tonsil	Epithelial	6½	None	21	22	3	11
MX OLC1	"	Cerebral cortex	Macrophage	5	"	19	22	4	11
MX OLC2	"	"	"	7	2½–3	20	29½	7½	10
MX OLC3	"	"	"	6½	None	19½	26½	5	>10
55 IV 2	"	Sarcoma	Sarcoma	1	"	13	25½	1	3½
78 IX R_4	"	Gingiva	Epithelial	None	—	—	—	—	25
76 IX R_2	"	Knee joint	Spindle	None	—	—	—	—	20

24 VIII L_1	"	HeLa	Malignant epithelial	9½	Obscure	13½	24	15	19
24 VIII L_2	"	"	"	7½	"	18	28	4	21
24 VIII L_3	"	"	"	8½	½–1	12	25	4	23
36 VIII L_4	"	"	"	12	Obscure	13	22½	4	19
37 VIII L_1	"	"	"	10	1½–2½	12½	22	5	18
70 VIII L_2	"	"	"	8	4–5	21	23	4	10
70 VIII L_3	"	"	"	7½	Obscure	12	28	4	14
75 VII R_1	"	"	"	8	1½–2	11½	26	5	25
67 VII R_3	"	"	"	9½	3–4	20½	29	5	14
6 IX R_2	"	"	"	8½	2–3½	11½	27	3	21
72 IX R_2	"	"	"	39	Obscure	19½	23	8	61
23 X L_3	"	"	"	4½	2½–3½	11	23	Abnormal	Abnormal
23 X L_4	"	"	"	7	None	15½	22½	6	28
44 X L_4	"	"	"	8½	3–3½	14	24	6	15
28 XI R_1	"	"	"	10	1–1½, 2–2½	15	21½	4	16
34 XI R_1	"	"	"	14½	Obscure	22	23	5	16
67 VIII L_4	Rat	Hypophysis	Spindle	6½	"	20	28	5	9
42 IV 5	Monkey	Cerebral cortex	Macrophage	5½	2–2½	17	24½	1	8
42 IV 6	"	"	"	8½	Obscure	19½	24	2	18
47 X L_5	Cat	Cerebellum	Pial	5–6	—	—	—	6	7



TEXT-FIGURE 1.—Anaphase and cleavage of a cell from a culture of monkey cerebral cortex (sequence 42 IV 5). Solid line representing anaphase movements was made by measuring the distance between the opposite centromeric groups at half-minute intervals. Dotted line representing cleavage was made by measuring the cell width at equatorial plate at 1-minute intervals. Anaphase curve shows an apparent period of pause.

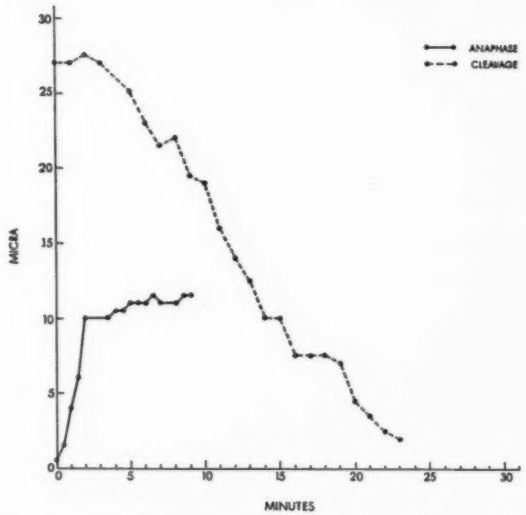


TEXT-FIGURE 2.—Anaphase and cleavage of a cell from a culture of human testis (sequence 57 VIII L_4). Solid line representing anaphase movements was made by measuring the distance between the opposite centromeric groups at half-minute intervals. Dotted line representing cleavage was made by measuring the cell width at equatorial plate at 1-minute intervals. Anaphase curve shows no apparent period of pause.

coefficient between the final centromeric distance and the time for anaphase among the 16 HeLa cells was 0.117, and that between this distance and the width of the cells was 0.091, indicating that in each case there was no relation between the two factors.

The pattern of the anaphase movements in the HeLa strain was more diversified than that seen in the other material. In some cases the chromosomes separated very rapidly at first, reaching a distance of 11 or 12 μ within 2 or 3 minutes, and stopped moving any further or moved very little (text-fig. 3). In others the movements may have been slow but were more regular in their rate of migration.

Table 1 also contains data regarding cells of special interest. For example, sequence 47 X L_5 provided a record of the nuclear events during the entire prophase. The record was made of a pial cell from the cerebellum of a newborn kitten photographed by Dr. H. Felton and Dr. A. Gaggero. At the beginning of the sequence the nucleus appeared to be rather homogeneous in structure except for the nucleolus, which showed very high density with phase optics. The nucleolus had two major irregular branches, but no other bodies of high contrast were noted (fig. 1). In approximately 7 minutes the nucleolus produced a small side branch, first appearing as a knoblike projection near the base of one of the original



TEXT-FIGURE 3.—Anaphase and cleavage of a cell from the HeLa strain (sequence 6 IX R_2). Solid line representing anaphase movements was made by measuring the distance between the opposite centromeric groups at half-minute intervals. Dotted line representing cleavage was made by measuring the cell width at equatorial plate at 1-minute intervals. Anaphase curve shows an early rapid movement of the chromosomes but very little movement after the first 2 minutes. Probably there was little spindle elongation in this cell. Not all the cells of the HeLa strain behaved in this way.

branches. Our failure to observe the presence of chromonemes was probably due to the fact that at that stage of prophase the chromonemes were still markedly hydrated and therefore did not show distinct contrast. The newly formed side branch of the nucleolus constricted near its base (fig. 2) 5 minutes later and then it lengthened to approximately 5 μ. Meanwhile, a second small branch had developed by the side of the first one (fig. 3). Both branches broke off from the nucleolus (fig. 4) and the pieces were seen to wander in the nucleus for a considerable time before they slipped out of focus (fig. 5).

It is known that large heterochromatic aggregates are liable to be broken down into smaller units, each of which appears to form a part of an individual chromosome (*8*). Since in this cell there was only one large nucleolar body, it probably represented both the nucleolus and pieces of heterochromatin attached to it. The nucleus rotated and, during this stage, many small branches were produced and detached from the nucleolus. Approximately 35 minutes after the beginning of this sequence chromosomes could be clearly defined, and the nucleolus, which had been constantly changing its shape, became smaller, with a dense periphery and a pale center showing much lower density (fig. 6).

The last piece of the nucleolus to fragment is shown in figures 7 (57 minutes) to 11 (74 minutes). This broken piece was probably a true chromosome because at one stage it clearly showed two long strands. It moved about in the nucleus while the main nucleolar mass lost its high optical density, and the chromosomes were very prominent (fig. 12).

The disappearance of both the nucleolus and the nuclear membrane occurred at approximately the same time. It would seem, however, that the disintegration of the nuclear membrane preceded slightly that of the nucleolus, for when the last trace of the nucleolus was still observable the chromosomes made a sudden convergence toward the center as if they were pushed by a force applied evenly upon the periphery of the "nucleus." Figures 13 and 14 represent two successive stages of these processes. The true prometaphase (fig. 15) followed immediately, and the process of metakinesis took approximately 10 minutes, after which metaphase arrangement was achieved (fig. 16). It can be seen from the figures that the rounding up of the cell actually began at prometaphase.

Metaphase lasted for only 13 minutes and then anaphase started. The plane of division was an oblique one and was not suitable for analysis. Anaphase required 5 to 6 minutes. The cleavage process, beginning at the last minute of anaphase, required approximately 7 minutes. The daughter cells were recorded until they completed their reconstitution; the Flemming body between the daughter cells was observed for more than 112 minutes before it was pulled out of the photographic field by one of the daughter cells.

Three examples of abnormal division were provided from records of cultures of a synovial joint, a fibrosarcoma, and a pigmented gingiva.

Sequence 76 IX R_2, photographed by Dr. Hugh Clark from a culture of human synovial knee joint, provided interesting phenomena of dynamic

cytology. The film record began with a polar view of the metaphase. The chromosomes moved about, presumably with the usual oscillation on the spindle, but the plate remained unchanged for approximately 4 hours. After this the spindle made a turn of 90° and the poles were then located at east to west position on the projection screen. The metaphase plate after this stage remained in side view. The rotation of the spindle apparatus took approximately 35 minutes, but 10 minutes later another 90° twist of the spindle took place and the poles were oriented at north to south position. The position of the spindle changed a third time by about 45° and the poles were set at north-east to south-west position. The chromosomes were then highly condensed and were arranged on a perfect plate. This stage lasted for about an hour, when another rotation occurred which turned the spindle nearly 360° and left the plate facing east to west. The poles soon changed their positions again, moving north-west to south-east and then due north to south by gradual spindle shifts. There were no further changes in the next 6 hours.

During all these movements of spindle orientation there was little change in the morphology of the chromosomes. The chromosomes appeared to be superspiralized bodies, each with two distinct chromatids and a centromeric constriction. Before the cell eventually divided, however, the chromosomes appeared to be more fuzzy in outline and seemed to be clumping together. Cleavage took place a little more than 11½ hours after the initial frame, with the chromosomes still on the metaphase plate. Apparently there was no anaphasic movement. At least some chromosomes were definitely included in one of the daughter cells without separation, and the daughter cells assumed a normal flattened appearance after much violent bubbling.

The cell in sequence 55 IV 2, taken from a 6-day-old culture of human subcutaneous fibrosarcoma, behaved in a manner similar to that of the synovial joint in that there was no apparent anaphase. The cytoplasmic cleavage cut through the metaphase plate forming two daughter cells. The chromosomes, lying at the equatorial sides of the daughter cells, soon despiralized and formed interphasic nuclei. However, unlike the previous cell, in which a number of chromosomes were included in one of the daughter cells without separation, it seemed that the cytoplasmic cleavage of this cell did separate most of the chromatids equally. Film analysis of this sequence proved that the chromosomes had moved approximately 8 μ apart in 1 minute when cleavage cut through the metaphase plate. Therefore the cleavage process of this cell was prematurely superimposed on anaphasic movement.

Sequence 78 IX R_4 was photographed by Dr. M. Cattoni from a culture of human pigmented gingiva. The chromosomes at the stage when the movie began were arranged on a plate which presented a side view. They were long and slender as if they were in the first meiotic metaphase. This was especially well illustrated by the long chromosomes with submedian centromeres. The centromeres of the two chromatids were far apart and were pointing to the opposite poles as in anaphase but the tips of

the arms remained associated. The ends of the chromatids of longer arms paired for a considerable distance. For 3½ hours after the motion-picture record began the chromosomes oscillated up and down the spindle, lengthening and shortening like elastic bands, without any sign of division.

No anaphase was observed, but a nuclear membrane began to form before the chromosomes showed any uncoiling. If cleavage had not occurred the whole process might be considered as a modified meta-reduplication [for terminology see (*9*)]. However, the cell constricted at the middle and thus cut the newly formed nucleus in two within approximately 25 minutes. Many chromosomes were distinctly observed to be included in one of the two daughter cells without separation, and these developed complete nuclear membranes.

Discussion

It has been emphasized by many previous workers (*9–11*) that time-lapse phase cinematography can reveal many dynamic features of cellular activities which by microscopic observation alone might escape notice. In the case of anaphase and cleavage movements, although still photography suffices, motion-picture technique gives a more precise and permanent record for each cell.

The data presented in this article on anaphase movements of mammalian cells confirm the findings of Ris, Boss, and other workers on lower animals. In some cases there was a definite two-step pattern of chromosome movement during anaphase. However, the lack or obscurity of the pause period in some of our sequences was not surprising. Our reading of half-minute intervals may not have been as revealing as the finer intervals used by other authors. Ris also used half-minute intervals, but his insect material was maintained at a temperature not higher than 30° C., hence the movements were much slower. Since it is known that chromosome movement and spindle elongation may overlap and may not be in the same proportion in different types of cells (*3*, *12*) and since some of our sequences did show the obvious pause, we consider that the two-step anaphase movement also exists in mammalian material.

Boss (*5*) found that in amphibian material the beginning of cleavage coincides with the beginning of the second period of anaphase and the period of spindle elongation. This was not always found in our records of mammalian cells. Usually the pause period occurred 2 or 3 minutes after the onset of anaphase, but cytokinesis started 1 or 2 minutes later. In extreme cases cytokinesis might begin after the chromosomes had reached the poles. Equally variable was the end stage of cleavage. In certain cases cleavage might be completed before the chromosomes had shown any sign of despiralization, yet in others cleavage might be completed after the nuclear membranes of the daughter cells had been formed. It is probably unfortunate that previous workers have used cleavage as one of the principal criteria in describing mitotic events. In our opinion the anaphase should be defined as the period beginning with the separation of the chromosomes at the equator and ending with the cessation of their poleward movements, regardless of the despiralization process.

Abnormal mitotic divisions are of special interest in the realm of dynamic cytology. Although such cells have been found repeatedly both in normal and in malignant tissues and both *in situ* and *in vitro*, how much abnormality found in cell cultures is due to the *in vitro* condition and how much information obtained from cell cultures can thus be used to interpret what happens in the body are questions which remain unresolved.

In a consideration of the problems of somatic aneuploidy it was pointed out (*13*) that cells from cultures not derived from strains under continuous cultivation probably are very similar to those *in vivo*. Using his ingenious method for studying epidermal cells, Algire (*14*) demonstrated that many cell activities observed from tissue-culture materials also behaved in a similar way in the *in vivo* situation. Critics may attack his experiments with the argument that the skin cells were stretched in the chamber and therefore they were no longer normal. Interpretations of cell activities based on fixed preparations are static and even more indirect; yet they have been taken for granted as providing classic answers. Because cells in culture are, after all, living systems they metabolize in a normal way; they exhibit many cell functions performed also in whole organisms and undergo cell division almost indefinitely. Moreover, thousands of dividing cells have been observed by many competent workers in culture preparations, and abnormal divisions were seen to occur only occasionally in nonmalignant tissues. It does not seem reasonable to attribute to the *in vitro* situation more than a fraction of the many kinds of abnormalities seen.

For cinematographic studies of cell activities, however, one factor should be kept in mind—the illuminating system employed for photography may damage living systems after prolonged use. Cells in metaphase are probably most vulnerable. Therefore when a dividing cell suddenly dies it may or may not be due to internal causes. However, on the basis of abnormal mitosis which we have analyzed in cultures of the HeLa strain [(*15*); Moorhead and Hsu, unpublished data], both in the living state and in fixed preparations, there was no significant difference between the two sets of data. Moreover, many abnormal mitotic figures, such as the meiosis-like plate of the human gingiva reported here, were already abnormal upon being located. It is reasonably safe to conclude that those abnormalities were not caused by light injury.

Judging from the appearance of the metaphase chromosomes of the gingiva cell, that of the "d-mitotis" in some mouse ascites tumors (*16*) and the sticking chromosomes in some human cancers (*17*), it appears that all represent the same type of anomaly. Whether all such cells would behave as did the gingival element is not known because other authors studied fixed cells. At any rate the present case has an interesting relation to the problem of amitosis. The division of the nucleus by cleavage was similar to the so-called amitosis; yet it was apparently preceded by mitosis up to the metaphase stage. The term "pseudo-amitosis" has been employed to describe the types of cells which possess telophasic bridges connecting the two daughter nuclei, presenting the

appearance of a constricted nucleus. In our opinion the case of the gingiva cell is best described by this term.

Summary

Anaphase and cleavage movements of 36 mammalian, including 32 human, cells in tissue culture were studied with the aid of phase-cinematographic technique.

One sequence of a cat pial cell showed complete mitosis at high magnification. In early prophase the nucleolus produced branches and shed them into the nucleus, which was rotating actively. The nucleolus became paler (low optical contrast) in the center as the prophase developed, and the dissolution of the nuclear membrane seemed to occur immediately before that of the nucleolus.

A cell from a culture of human pigmented gingiva showed the formation of the nuclear membrane after prolonged metaphase without anaphasic disjunction. Cytoplasmic cleavage cut through the newly formed nuclear membrane and formed two daughter cells.

A cell from a culture of human synovial knee joint showed that cytokinesis may occur at metaphase, and another cell from a human sarcoma showed that anaphase and cleavage may take place almost simultaneously.

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PLATE 56

Early mitosis of pial cell in culture of cerebellum of newborn kitten. Time interval (in minutes) from preceding stage marked at upper left corner of each figure. For magnification see scale on figure 1.

FIGURE 1.—Beginning of the sequence. Note the nucleolus, its high contrast and irregular shape.

FIGURE 2.—The nucleolus has formed a side branch (*see arrow*).

FIGURE 3.—The side branch has reached 5 μ in length and a small additional branch is formed at its side.

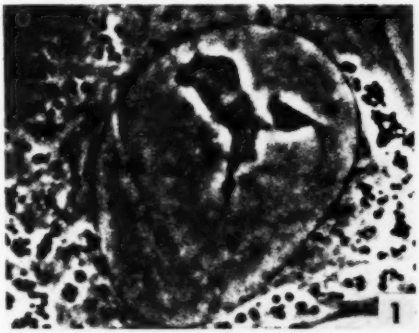
FIGURE 4.—The long branch is broken (*see arrow*).

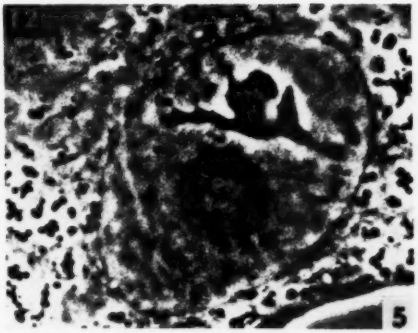
FIGURE 5.—The nucleus has shown rotary activity and the nucleolus has changed its form. The broken piece is now at the side of the nucleus (*see arrow*).

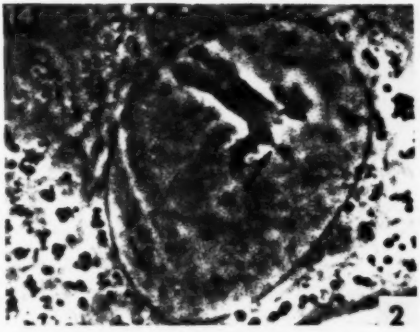
FIGURE 6.—Nucleolus has become smaller and paler in center. Distinct prophase chromosomes.

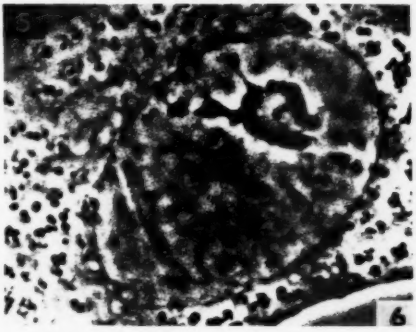
FIGURE 7.—Prophase. Periphery of nucleolus has become darker.

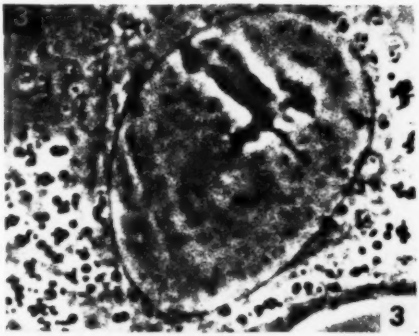
FIGURE 8.—Contraction of the branches of the nucleolus. Note the branch indicated by an *arrow*.

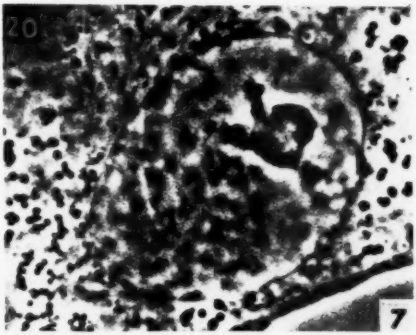


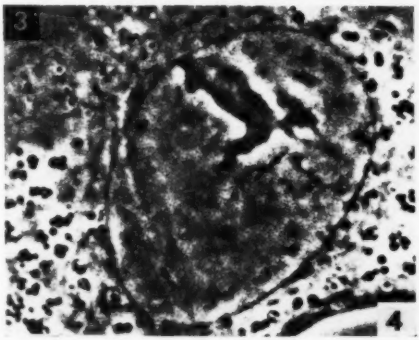












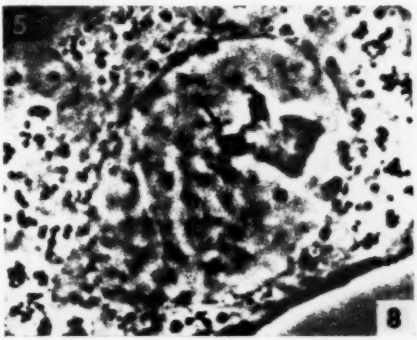


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PLATE 57

Continuation of series illustrated on Plate 56.

FIGURE 9.—Lengthening of the branch of the nucleolus (*see arrow*).

FIGURE 10.—Further lengthening of the branch.

FIGURE 11.—The branch has broken from the nucleolus. *Arrow* points at the broken piece of nucleolus.

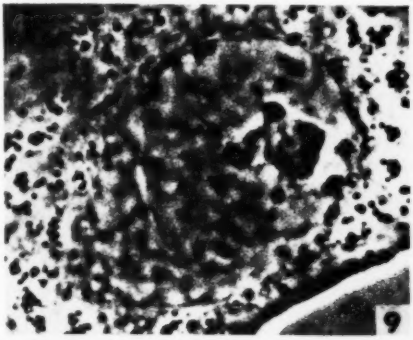
FIGURE 12.—Late prophase.

FIGURE 13.—The nucleolus has lost its optical density, while chromosomes are very dense; the nuclear membrane is disappearing.

FIGURE 14.—Early prometaphase. Nuclear membrane has disappeared while the last trace of nucleolus is still present.

FIGURE 15.—Metakinesis.

FIGURE 16.—The beginning of metaphase.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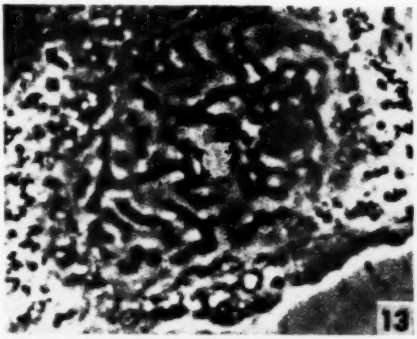
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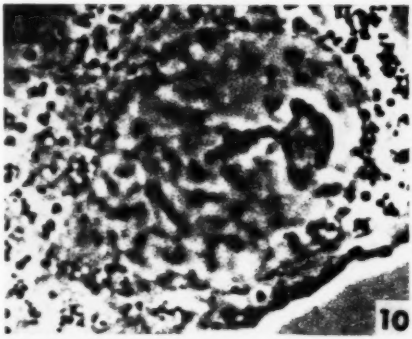
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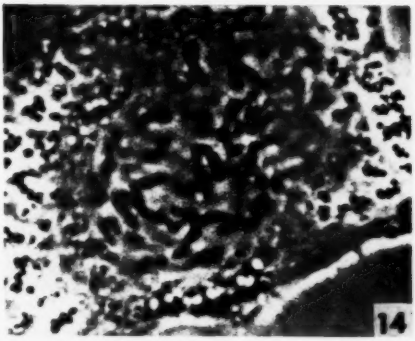
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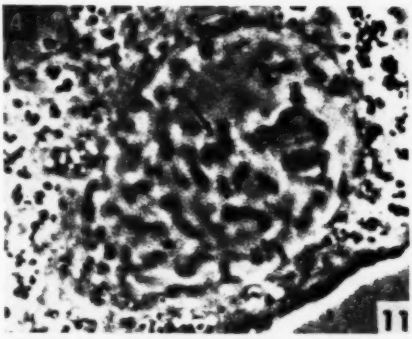
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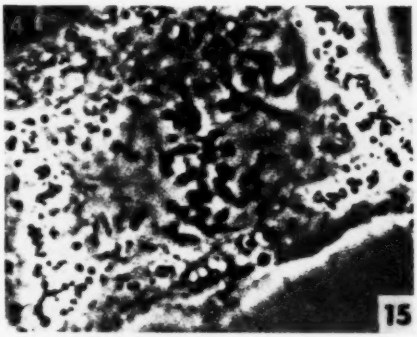
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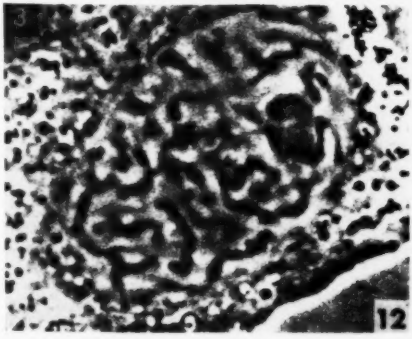
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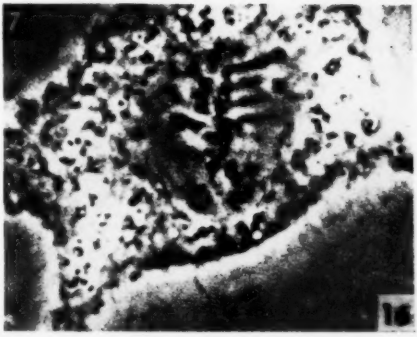
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Factors Influencing the Specificity of Action of an Antileukemic Agent (Aminopterin): Host Age and Weight[1]

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In the study of the chemotherapy of neoplasia, the interrelationships of the host, the tumor, and the drug are of fundamental importance. With respect to the drug, it was illustrated that factors such as the time of initial treatment, duration of treatment, total dosage, interval between the administration of individual doses, and the number of doses may influence the host-tumor relationship (*1–3*). This relationship may also be influenced by using a metabolite with an antagonist (*2, 4, 5*).

It was the aim of the current investigation to determine the influence of the age of the host on the host-tumor-drug relationship in chemotherapy. The influence of the age of the host was studied in leukemic mice with respect to:

(*a*) the lethality to the host of aminopterin, administered alone or with citrovorum factor (CF);

(*b*) the susceptibility of the host to the growth and lethal action of the tumor;

(*c*) the sensitivity of the tumor to treatment with aminopterin alone or administered with CF;

(*d*) the antileukemic specificity of action of aminopterin, administered alone or with CF. This provides a measure of the relative benefits to be obtained through the use of aminopterin treatment (*4*).

The indirect influence of the age of the host was also considered. To what extent does age influence the above responses through its effect on weight and to what extent is its influence direct?

Methods

The experiments were conducted with the transplantable lymphoid leukemia 1210 carried in DBA/2J, (A/LN $\times$ DBA/2J)F_1, and (BALB/cAn $\times$ DBA/2J)F_1 hybrid male mice. Stock tumor was carried in strain DBA/2J male mice. The animals were inoculated in the right thigh with 0.1 ml. of a saline suspension of tumor cells of designated concentration. Aminopterin was prepared in 2 percent sodium bicarbonate. CF was

[1] Received for publication June 1, 1955.

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prepared in distilled water.[3] The mice were randomized within the individual age groups. The drugs were administered subcutaneously in the left axillary region in the constant volume of 0.01 ml. per gm., and the order of their injection was randomized. Leukemic mice not receiving the drug were observed for survival time. Where the leukemic mice were treated with drug, the day of death provided a basis for separating mice that died of drug toxicity from mice that succumbed to growth of the tumor. The detailed experimental and statistical procedures have been described previously (*4*).

Results

Table 1 and text-figures 1 to 3 summarize the results of the first experiment in this investigation.

In this experiment, 6-week-old (A/LN × DBA/2J)F_1 male mice were compared with 11- to 12-week-old mice. Four days following implantation of L1210 tumor cells, mice in each age group were treated with graded doses of aminopterin, with and without concomitant CF. These mice were then observed for lethal effects of the treatment and for time of death from tumor.

The greater sensitivity of the younger mice to the drug is readily seen by comparing the LD50's. The aminopterin LD50 for the young mice was 2.7 mg. per kg. as compared with 11.8 mg. per kg. for the older mice. Administration of 15 mg. per kg. of CF concomitantly with aminopterin raised the aminopterin LD50 for the young mice to 32 mg. per kg. and for the older mice to 51 mg. per kg. This effect of age on sensitivity to aminopterin was previously observed in mice without tumor (*6*).

Text-figure 1 shows the fitted lines relating the average survival times of mice dying from tumor to the dose of aminopterin administered. It may be noted that when equal doses of aminopterin were given the older mice survived for a longer period of time than did the younger mice. However, when adjustment is made for the longer survival time of the older untreated controls, the lines for the two age groups coincide fairly well (text-fig. 2). Apparently, then, the age of the leukemic mouse does not alter the sensitivity of the tumor to aminopterin treatment. The coincidence of the adjusted concomitant CF lines further suggests that the age of the host does not alter the effectiveness of CF in protecting the tumor against the action of aminopterin.

In text-figure 3, the fitted survival-time curves are replotted, this time against the probit of mouse mortality due to aminopterin. It may be noted that for both young and old mice concomitant administration of CF served to reduce the average survival time. This reduction of survival time indicates that concomitant administration of CF provided proportionately greater protection for the tumor than for the host, in both young and old mice, against the action of aminopterin (*4*). This text-figure indicates that, on the average, following aminopterin treatment (with or without concomitant CF), for given cost in lethality from drug, it took the

[3] Aminopterin and citrovorum factor were kindly provided by Dr. James M. Smith, Jr., of the Calco Chemical Division of the American Cyanamid Company, Bound Brook, N. J.

TABLE 1.—*Host and tumor response to aminopterin with and without concomitant administration of citrovorum factor. Young and old (A/LN × DBA/2J)F_1 male mice*

Drug deaths shown are among 10 treated mice; *P*=calculated probit of toxic mortality*; S. T. /N=average survival time in days for mice surviving drug toxicity but dying from tumor/number of mice on which this average is based; leukemic inoculum=78,000 cells per mouse; treatment administered 4 days following tumor inoculation.

Aminopterin dose (*mg./kg.*)	Aminopterin only						Aminopterin + 15 mg./kg. citrovorum factor					
	Young mice (6 weeks)			Old mice (11–12 weeks)			Young mice (6 weeks)			Old mice (11–12 weeks)		
	Drug deaths	*P*	S. T. /N	Drug deaths	*P*	S. T. /N	Drug deaths	*P*	S. T. /N	Drug deaths	*P*	S. T. /N
0.25	0	−0. 35	14.6/10									
0.42	0	0. 81	15.6/10									
0.69	0	1. 96	16.3/9†									
1.16	0	3. 12	16.4/10									
1.94	2	4. 27	17.9/8	0	0. 91	18.9/10						
3.2	8	5. 43	19.0/2	1	2. 07	18.9/9	0	−0. 20	14.6/10			
5.4	9	6. 59	17.0/1	0	3. 22	20.5/10	0	0. 95	15.6/10			
9.0	10	7. 74	—	1	4. 38	21.6/9	0	2. 11	15.7/10	0	1. 08	17.0/10
15.0				8	5. 53	20.0/1‡	0	3. 27	16.0/10	0	2. 23	18.1/10
25.0				9	6. 69	21.0/1	6	4. 42	16.2/4	0	3. 39	18.7/10
42				10	7. 85	—	4	5. 58	16.3/6	1	4. 54	18.4/8§
69				10	9. 00	—	10	6. 74	—	10	5. 70	—
116							10	7. 89	—	10	6. 86	—
193										10	8. 01	—
322										10	9. 17	—
536										10	10. 33	—
	LD50=2.7 mg./kg.			LD50=11.8 mg./kg.			LD50=32 mg./kg.			LD50=51 mg./kg.		

Controls: 30 young mice in various control groups all died of tumor between the 12th and 17th day following tumor inoculation; average=14.3 days.

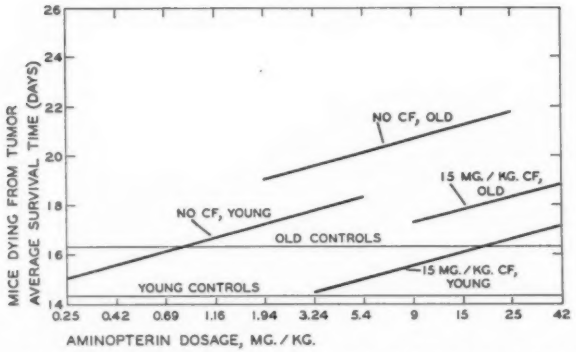
40 old mice in various control groups all died of tumor between the 14th and 25th day following tumor inoculation; average=16.4 days.

*Calculated probits obtained from the fitted maximum likelihood dose-response curves. A 4-way common probit slope was fitted to the four groups employing one cycle of the Cornfield-Mantel procedure (*12*).

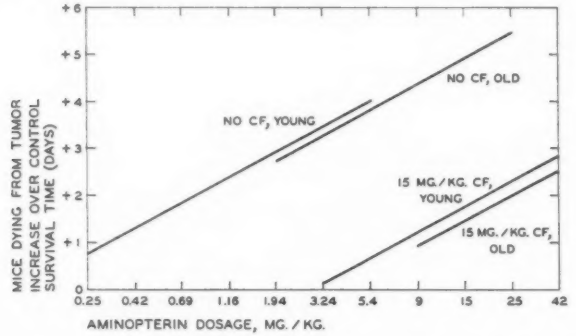
†One mouse missing from this group on day 12.

‡One mouse in this group dead of extraneous causes on day 13.

§One mouse apparently tumor-free in this group at 60 days.



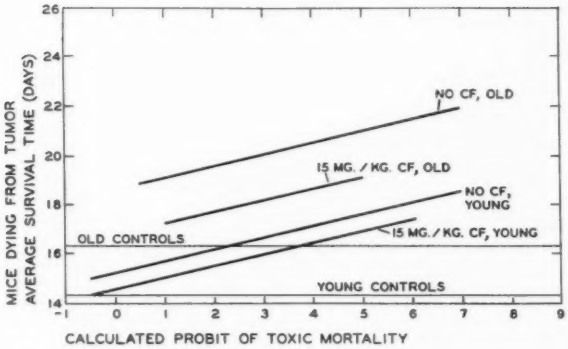
TEXT-FIGURE 1.—Effect of age of host on the survival time-aminopterin dosage relationship in leukemic mice. The aminopterin was administered alone and with citrovorum factor (table 1). Fitted common slope least-squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the logarithm of the dose of aminopterin are plotted.



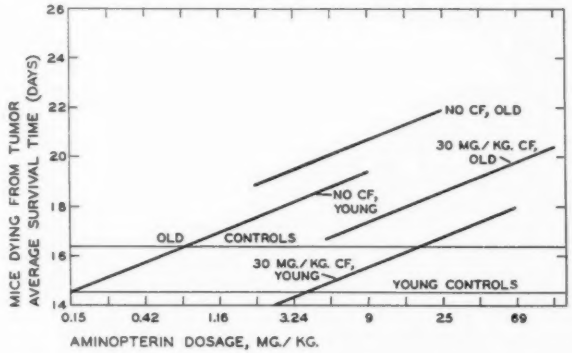
TEXT-FIGURE 2.—Effect of age of hosts on the increase in survival time of leukemic mice given different doses of aminopterin over controls. Aminopterin is administered alone and with citrovorum factor (table 1).

tumor about 3 days longer to kill the older mice than the younger mice. This 3-day advantage for the older mice is attributable to their lesser susceptibility to the lethal action of the tumor and to their lesser sensitivity to aminopterin. The latter permitted the use of heavier treatment.

As a check on this experiment, a parallel experiment was performed, this time on 6- and 18-week-old (BALB/cAn × DBA/2J)F_1 male mice. The results of the parallel experiment are summarized in table 2 and text-figures 4 to 6. In this experiment, the aminopterin LD50 for the young mice was 6.2 mg. per kg. and 29 mg. per kg. for the 18-week-old mice. These were raised to 41 mg. per kg. for the young mice and 122 mg. per kg. for the older mice by the concomitant administration of 30 mg. per kg. of CF. The results of this experiment are essentially similar to those of the first, the older mice again showing about a 3-day total advantage over the younger mice at fixed cost in lethality of treatment.



TEXT-FIGURE 3.—Effect of age of host on the survival time-toxic mortality relationship in leukemic mice. The aminopterin was administered alone and with citrovorum factor (table 1). Fitted common slope least-squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the calculated probit of toxic mortality from aminopterin are plotted (table 1).



TEXT-FIGURE 4.—Effect of age of host on the survival time-aminopterin dosage relationship in leukemic mice. The aminopterin was administered alone and with citrovorum factor (table 2). Fitted common slope least-squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the logarithm of the dose of aminopterin are plotted.

To investigate the nature of the effect of age on susceptibility to tumor, a third experiment, summarized in text-figures 7 and 8, was conducted. The purpose of this experiment was to determine whether age affected only the time to death from tumor or whether it also affected the probability that a transplanted tumor would "take" successfully.

For this purpose, greater variation in the age groups of the mice to be compared was necessary. Six-week-old, 13- to 14-week-old, and 41-week-old (BALB/cAn×DBA/2J)F_1 male mice received graded inoculum levels of leukemia cells, ranging from 29 cells to 2.28×10^6 cells per mouse. Text-figure 7 shows the percentage mortality from tumor at each inoculum level for each of the three age groups of mice. In total, of eighty mice in each

TABLE 2.—*Host and tumor response to aminopterin with or without concomitant administration of citrovorum factor. Young and old (BALB/cAn × DBA/2J)F_1 male mice*

Drug deaths shown are among 10 treated mice; *P*=calculated probit of toxic mortality*; S. T. /N=average survival time in days for mice surviving drug toxicity but dying from tumor/number of mice on which this average is based; leukemic inoculum=104,000 cells per mouse; treatment administered 4 days following tumor inoculation.

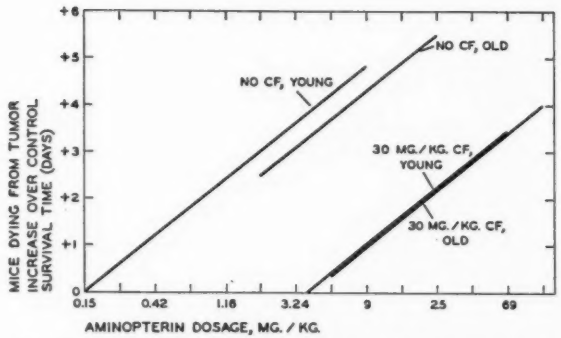
Aminopterin dose (*mg./kg.*)	Aminopterin only						Aminopterin+30 mg./kg. citrovorum factor					
	Young mice (6 weeks)			Old mice (18 weeks)			Young mice (6 weeks)			Old mice (18 weeks)		
	Drug deaths	*P*	S. T. /N	Drug deaths	*P*	S. T. /N	Drug deaths	*P*	S. T. /N	Drug deaths	*P*	S. T. /N
0.15	0	−0. 33	14. 8/10									
0.25	0	0. 40	15. 8/10									
0.42	1	1. 13	15. 4/9									
0.69	0	1. 87	16. 4/10									
1.16	0	2. 60	17. 3/10									
1.94	1	3. 33	17. 1/9	0	1. 12	18. 3/10	0	0. 62	14. 9/10			
3.2	1	4. 06	17. 6/9	0	1. 85	18. 0/10	1	1. 35	14. 3/9			
5.4	2	4. 80	18. 6/8	0	2. 59	20. 2/10	0	2. 09	14. 9/10	0	0. 53	17. 2/10
9.0	7	5. 53	19. 7/3	0	3. 32	21. 6/10	0	2. 82	15. 1/10	0	1. 26	16. 7/10
15.0				1	4. 05	22. 6/8†	0	3. 55	15. 0/10	0	2. 00	17. 9/10
25.0				1	4. 78	21. 8/9	1	4. 28	16. 9/9	0	2. 73	18. 4/10
42				10	5. 52	—	5	5. 02	17. 8/5	0	3. 46	18. 4/10
69				10	6. 25	—	7	5. 75	18. 7/3	0	4. 19	21. 3/9‡
116				10	6. 98	—	10	6. 48	—	4	4. 93	20. 0/6
193							10	7. 21	—	10	5. 66	—
322										10	6. 39	—
536										10	7. 12	—
	LD50=6.2 mg./kg.			LD50=29 mg./kg.			LD50=41 mg./kg.			LD50=122 mg./kg.		

Controls: 30 young mice in various control groups all died of tumor between the 13th and 16th day following tumor inoculation; average=14.6 days.
30 old mice " " " " " " " " " " 15th and 18th " " " " " =16.4 days.

*Calculated probits obtained from the fitted maximum likelihood dose-response curves. A 4-way common probit slope was fitted to the four groups employing two cycles of the Cornfield-Mantel procedure (*12*).

†One mouse apparently tumor-free in the group at 97 days.

‡One mouse killed accidently in this group on day 16.

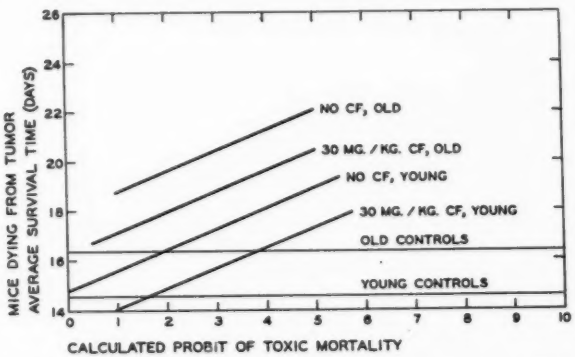


TEXT-FIGURE 5.—Effect of age of host on the increase in survival time over controls in leukemic mice with respect to aminopterin dosage, when the aminopterin is administered alone or with citrovorum factor (table 2).

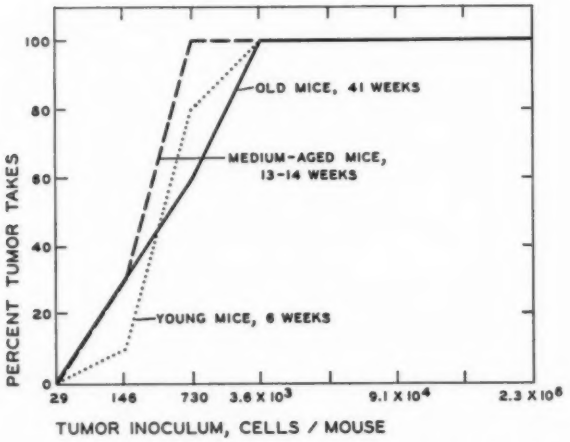
age group, fifty-nine 6-week-old mice, sixty-three 13- to 14-week-old mice, and fifty-nine 41-week-old mice died from tumor. There is no significant indication that the age of the mouse is a factor in determining the probability that a tumor transplant will "take" successfully. However, in text-figure 8 it may be noted that, for mice that did die of tumor, at a given level of leukemic inoculum, the older the mouse the longer the survival time. For each of the three age groups of mice, as the inoculum level diminished, the survival time increased.

This experiment would suggest that the success of transplantation is not influenced by the age of the mouse, but does depend on the level of inoculum employed. The length of time it takes the tumor to kill depends on both the level of tumor inoculum and the age of the mouse.

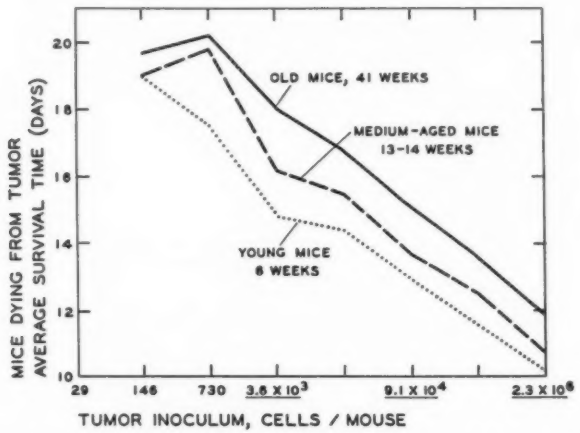
To rule out any possibility that the effect of the age of the mouse on the survival time of the leukemic mice might be attributable to genetic factors



TEXT-FIGURE 6.—Effect of age of host on the survival time-toxic mortality relationship in leukemic mice. The aminopterin was administered alone and with citrovorum factor (table 2). Fitted common slope least-squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the calculated probit of toxic mortality from aminopterin are plotted (table 2).



TEXT-FIGURE 7.—Effect of age of mouse on the percentage mortality (takes) following inoculation of leukemic cells. The percentage mortality is plotted against the logarithm of tumor-cell concentration for three age groups of mice.



TEXT-FIGURE 8.—Effect of age of mouse on the survival time following inoculation of leukemic cells. The survival time is plotted against the logarithm of tumor-cell concentration for three age groups of mice.

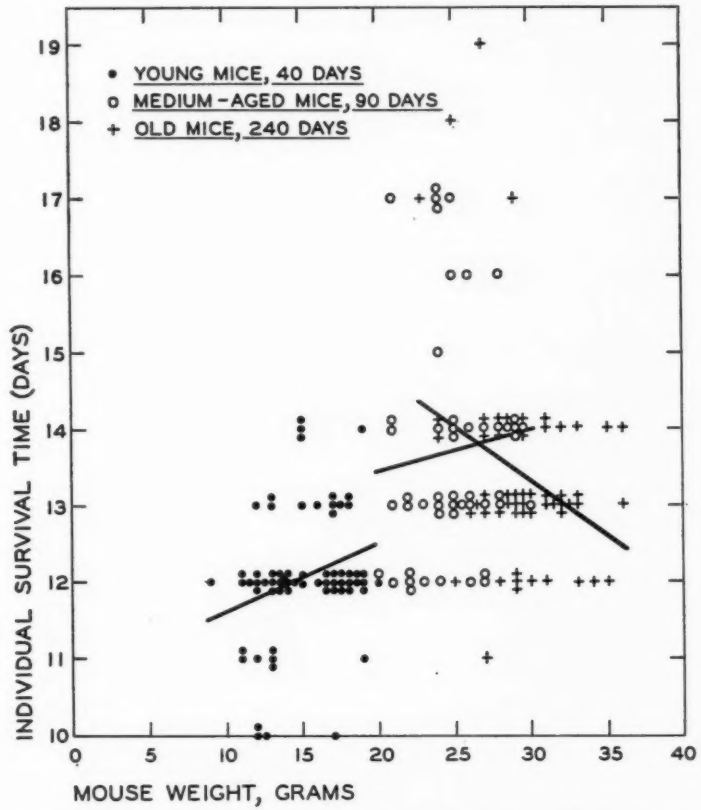
in the employment of F_1 hybrids, the observation was checked in DBA/2J mice. In DBA/2J mice too, for a given inoculum, older mice survived for a longer period of time than younger mice. With 2,000,000 cells in DBA male mice, 21-week-old mice gave a survival time of 12.4 $\pm$ 0.2 days, while 5-week-old mice gave a survival time of 11.1 $\pm$ 0.1 days. The possibility that the manner of caging the mice may have contributed to the difference in survival time between old and young mice was also ruled out. The difference was noted whether the mice were caged in groups or individually.

The effect of age on the survival time of the leukemic mice is apparently not characteristic of the route of inoculation of the tumor cells. Old mice survived longer whether the inoculum was introduced intramuscularly or intraperitoneally. However, for both old and young mice the survival time was reduced for the intraperitoneal route as compared with the intramuscular route.

Similarly, in other experiments with sarcoma 37, older mice survived for a longer period than younger mice (*7*).

The final experiment was conducted to determine the extent to which the factor of increased body weight could account for the longer time to death from tumor for the older mice. Three age groups of mice were employed, and the results are summarized in text-figure 9.

It was desirable in this experiment to secure maximum overlapping and spread in the weights of the mice in the three age groups. In addition to a random selection of mice from each age, the very small and very large



TEXT-FIGURE 9.—Effect of body weight on the survival time of leukemic mice, for three age groups of mice. Separate least-squares regression lines relating survival time to body weights are fitted for each of the three age groups.

remaining mice from those available were used to supplement the sample.

Six-week-old, 13-week-old, and 34-week-old (BALB/cAn × DBA/2J)F_1 male mice were implanted with 2.5×10^6 leukemic cells per mouse. The day of death of each individual mouse is plotted against its initial weight in text-figure 9. In addition, fitted least-squares lines relating time to tumor death to initial mouse weight are shown for each age group.

The comparison of the young mice and the mice of medium age was impaired by the failure to secure any overlapping in mouse weights for these two groups. Much of the difference in average time to death from tumor between these two groups can be accounted for by their average difference in body weight. About 1 day of the difference remains unaccounted for by weight and is just barely short of significance at the 5-percent level. There is thus a suggestion that age is a factor in increasing survival time over and above its indirect effect in accomplishing this through the accompanying increase in weight.

The average survival times for the medium and oldest groups of mice were quite close to each other. For the oldest group of mice, however, the average time to tumor death seemed to decrease, rather than increase, with weight. This decrease appeared to be attributable not so much to shortened survival times with extremely high weights, but rather to the lack of the occasional long survival times observed among mice of moderate size. Whether this is a characteristic of extreme weight, in general, or only for extreme weight among older mice could not be determined due to the absence of extreme weights in the younger age groups.

No experiments were conducted to determine the extent to which weight differences could account for differences in sensitivity of the young and old mice to aminopterin. The great differences in aminopterin LD50's for the young and old mice found in the first two experiments reported here would suggest that some real age factor may exist.

Discussion

In previous studies it was demonstrated that the antileukemic specificity of action of a drug, expressed in terms of the relative effect of the drug on tumor and host, is not fixed but may be altered by the manner in which the drug is employed (*1–5*). The current studies provide an illustration of how the antileukemic specificity of action of aminopterin may be influenced by the host. The antileukemic specificity of action of aminopterin was observed to be greater in older mice than in younger mice. At fixed cost in drug-dose mortality, aminopterin was more effective in increasing the survival time of older mice than younger mice. This advantage for older mice is attributable to two factors: (a) For a given level of tumor inoculum, without drug treatment, older mice survived longer than younger mice. (b) Older mice tolerated more drug than younger mice. Further investigation and analysis of these two contributing factors to increased antileukemic specificity of action showed the following:

a) Host-tumor relationship.—Age of host did not appear to influence the probability that a leukemic implant will "tkae" successfully. The prob-

ability of a successful "take" was a function of the tumor, increasing with the size of the inoculum. The duration of life of the host, when a "take" occurred, appeared to be a function of both the host and the transplant.

In general, the survival time was increased as the age of the host was increased and as the inoculum concentration was diminished. Part of the advantage of age in delaying time to death from tumor would appear to be attributable to the increased body weight of the older mice. Beyond a certain weight, however, the indication is that further increments in body weight may be of no additional advantage, or may actually result in some reversal of the original gain.

These observations are of interest with reference to the demonstration by Mider *et al.* (*8*) that the tumor is, in effect, a nitrogen trap. Dietary intake does not meet the requirement of the combination of host and tumor, and as a result, the needs of the tumor are supplied by the body stores, to the detriment of the host (*8–10*). Mider observed an increase in survival time of rats with Walker carcinoma 256 when the animals were fed a desiccated tumor preparation. Greater carcass weights and tumor weights were observed accompanying the increase in survival time (*11*). It is suggested that a comparable kind of situation may obtain with differences in initial body weight in the leukemic mice, the heavier mice having greater body stores of essential metabolites.

To what extent there may be a difference in the rate of growth and infiltration of the tumor in leukemic mice of different age and weight is difficult to determine. Preliminary studies indicate that the leukocyte count of the peripheral blood does not appear to rise so rapidly and the hemoglobin concentration does not appear to diminish so rapidly in old mice as compared with young mice. This does not necessarily mean that the tumor is growing more slowly in the old mice than the young mice. These are measurements per unit of blood and are not measures of the total peripheral blood leukocyte count or the total hemoglobin for the whole animal. If the tumors are multiplying at the same rate in young and old mice, the greater mass of the older, heavier mice would tend to "dilute out" any changes in the peripheral blood. That this may have occurred is indicated by the observation that immediately prior to death the rise in peripheral blood leukocyte count and the diminution in hemoglobin concentration appear to be the same for young and old mice. The suggestion is that the total number of leukemic cells achieved prior to death for an old, heavier mouse is greater than that required for a young, light-weight mouse. Although for equal inoculum there is some suggestion that the local tumor at the site of implantation grows somewhat more slowly in the old mice than in the young mice, the local tumor appears to achieve a larger size prior to death in older mice than in younger mice. Also, for equal survival time, accomplished by employing a larger inoculum in old mice than in young mice (text-fig. 8), the old mice have larger local tumors prior to death. The influence of age of the mouse on rate of growth and infiltration of tumor is under investigation.

b) *Host-tumor-drug relationship.*—The data indicate a measure of inde-

pendence of tumor and host with respect to drug treatment. The greater tolerance to aminopterin of the older mice as compared with the younger mice is not transmitted to the tumor. Thus, even though a specific dose of aminopterin was less toxic for an old mouse than for a young mouse, there was no diminution of the effect of the dose with respect to the tumor.

Similarly, with respect to the tumor, the effect of a specific dose of aminopterin administered with CF appears to be independent of the age of the host. In both old and young mice the CF reduced the toxicity of aminopterin for both host and tumor. However, the reduction in toxicity to the host was such that a specific dose of aminopterin remained less lethal for an old mouse than for a young mouse. The reduction of effect with respect to the tumor was such that a specific dose of aminopterin had the same effect in old and young mice.

Thus, by the employment of older mice, the toxicity of aminopterin (alone or with CF) for the host is effectively reduced, while the effectiveness of the drug with respect to the tumor is not altered. This permits the employment of higher doses of the drug in older mice, resulting in enhanced antileukemic effect at equivalent probit of toxic mortality.

In both old and young mice it is apparently more desirable to treat with aminopterin alone than with aminopterin plus concomitantly administered CF. Regardless of the age of the host, the concomitantly administered CF provided proportionately greater protection for the tumor than the host, thereby reducing the specificity of action of the aminopterin. It will be of interest to determine whether the enhanced antileukemic action observed on treatment with aminopterin in older mice with leukemia will be maintained on delayed administration of CF (*2*, *5*) and on multiple treatment schedules of aminopterin.

Summary

The antileukemic specificity of action of aminopterin was demonstrated to be influenced by the age of the mouse. At equal cost in drug-dose mortality, aminopterin was more effective in increasing the survival time of older mice as compared with younger mice. This increase was attributable to two factors: (*a*) Older mice survive longer than younger mice, following inoculation of an equal number of leukemic cells. At least part of the effect of age on the survival time is attributable to the initial weight of the mouse. The percentage "take" of the leukemia is not influenced by the age of the mouse. (*b*) Older mice tolerate a higher dose of aminopterin administered alone or concomitantly with CF than younger mice. The increased drug tolerance of the host is not transmitted to the tumor. This permits the employment of higher doses of aminopterin in older mice with a resultant increase in antileukemic action.

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Transplantable Adrenocortical Adenocarcinomas in Osborne-Mendel Rats Fed a Carcinogenic Diet[1]

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There are no published reports on adrenocortical carcinoma, either spontaneous or induced, in Osborne-Mendel rats and only a few reports on spontaneous adrenal tumors in any strain of rat have been found. Curtis *et al.* (*1*), in their statistical study of the lesions of old rats of several strains, report only 1 case of adrenal tumor in 31,000 rats examined. Yeakel (*2*) of the Wistar Institute observed medullary hyperplasia in 51 percent of males and 11 percent of females in a small group of 19 male and 18 female rats 2 years of age or more. Gillman *et al.* (*3*), from South Africa, report a very high incidence (85.5% in males and 76% in females) of pheochromocytomas in a line of Wistar strain rats. They examined 355 rats about 2 years of age or older. They described this lesion as a low-grade carcinoma. The present report describes adrenocortical adenocarcinomas observed in female rats of the Osborne-Mendel strain, aged 18 months or older, which were under observation for the development of liver lesions.

Materials and Methods

Sixty female rats of the Osborne-Mendel strain, about 4 months old, were divided into 2 groups of 30 each. They were fed a semisynthetic diet[3] containing 0.06 percent *p*-dimethylaminoazobenzene. This diet was the same as that used by Mulay and Firminger (*4*) except for modifications in the protein content. The first group of animals received 8 percent and the second group 6 percent casein in place of 12 percent in the original diet. Both groups of animals received the same treatment in all other respects. All animals were kept on this diet for 286 days, at the end of which time they were placed on a diet of Purina chow pellets. The animals were examined at weekly intervals for tumors. They were killed only when very sick or moribund. Fifty-five of 60 experimental animals were autopsied and all organs examined for macroscopic lesions. Pieces of liver, lungs, spleen, pancreas, kidneys, adrenal and pituitary glands, and

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] The composition of the semisynthetic diet was as follows: sugar, 82 or 84 percent; casein, 6 or 8 percent; corn oil, 5 percent; salt mixture, 4 percent; vitamin mixture, 1 percent. This diet had only 0.2 mg. percent riboflavine and, according to proposed standard for rat diet (*5*), was low in all vitamins of the B complex group. It contained no pteroylglutamic acid or tocopherol.

intestine were taken for microscopic examination, and sections were taken from any other organ which had a visible lesion or abnormality. These tissues were fixed in Zenker's acetic fluid and microscopic sections were stained with hematoxylin and eosin.

The adrenal tumors were transplanted to Osborne-Mendel rats, both male and female, about 2 months old. When the transplants were 2 to 4 months old, the host animals were killed and examined for changes induced by the growth of the transplant. Tissue from the transplanted tumor and representative organs of the host were prepared for microscopic study.

Results

The frequency of adrenal lesions in the experimental animals is recorded in table 1. The two groups of animals on 8 and 6 percent protein diets, respectively, were further subdivided by age into those above and below 18 months at the time of autopsy. Of the 13 animals under 18 months of age, 1 rat receiving the 6 percent protein diet showed an adrenocortical adenoma. Of the 42 animals over 18 months of age (18 to 30 months), 30 animals had adrenal tumors—an incidence of 71 percent. These tumors occurred in equal numbers of animals receiving 6 and 8 percent protein. Five rats of the 6 percent group and 7 rats of the 8 percent group, all over 18 months of age had no adrenal lesions. Thus there is no significant difference in the incidence of adrenal tumors in the two groups of animals, although one group received 1⅓ times as much protein as the other. Adrenocortical adenoma accounted for 26 of these lesions and adrenocortical adenocarcinoma accounted for the remaining 4. Thus the incidence of adrenocortical adenocarcinoma in 42 rats aged 18 months or older was 9.5 percent. Three of these cases were found in animals receiving 6 percent protein and one in animals receiving 8 percent protein. This gives an incidence of 15 percent for adenocarcinoma in the rats 18 months or older on the 6 percent protein diet. Figures 1 and 2 show the gross appearance of two of these primary adrenocortical adenocarcinomas. Metastases to the liver and lungs were observed in two cases.

TABLE 1.—*Number of rats with adrenal lesions tabulated according to age and protein intake*

	6 Percent protein		8 Percent protein	
	Under 18 months	Over 18 months	Under 18 months	Over 18 months
No lesion	6	5	6	7
All lesions	1	15 (75%)	—	15 (68%)
Adenocarcinoma	—	3 (15%)	—	1 (5%)

Three of these adrenocortical tumors and their metastases were transplanted into male and female Osborne-Mendel rats about 2 months old. These adrenal tumors, at the time of this writing, are in their 10th generation. They grow in 100 percent of cases. In 3 to 4 months, they reach

a size of about 3 × 4 × 4 cm. and a weight of 15 to 20 gm., and they metastasize to the liver and lungs. The gross appearance of one such transplant and its metastases to the liver and lung are shown in figures 3 and 4. Animals carrying this tumor transplant usually die in 4 to 6 months.

The actively growing adrenal-tumor transplant has a marked effect on the size and weight of the adrenals of the host. Relative weights of the adrenal glands of animals with the transplanted tumors and of normal animals of comparable age, sex, weight, and strain are recorded in table 2, and their relative sizes are shown in figure 16. The average weight of 15 adrenals from 8 animals bearing adrenocortical tumor is 11.2 mg., while that of 16 adrenal glands from 8 animals of comparable age and sex is 24.9 mg. This is a reduction in weight of over 50 percent. No striking changes were observed in any other endocrine organs of the host.

TABLE 2.—*Weight of adrenal glands from rats with transplanted adrenocortical adenocarcinoma and from normal controls*

Rats with adrenocortical adenocarcinoma		Normal rats	
Right adrenal (*mg.*)	Left adrenal (*mg.*)	Right adrenal (*mg.*)	Left adrenal (*mg.*)
13. 6	13. 8	31. 2	31. 0
13. 8	13. 6	25. 0	19. 2
11. 2	11. 0	19. 2	22. 2
15. 0	13. 4	34. 8	23. 4
8. 4	9. 2	16. 8	23. 6
12. 1	11. 6	30. 8	24. 8
11. 0	10. 0	21. 4	24. 0
11. 0	Primary tumor	28. 0	23. 2
Average 11.2 mg.		Average 24.9 mg.	

Histology of the Lesions

Adenomas.—The adrenal glands of 55 rats were examined microscopically, and 26 of these animals were found to have adenomatous lesions of the cortex in one or both glands. These lesions, designated as adenomas, consisted of circumscribed areas which were usually confined to the zona fasciculata. Some of these lesions were microscopic in size while others nearly replaced the gland and caused gross enlargement (fig. 5). The smaller nodules caused some compression of tissues around the periphery. The cells within them resembled cells of the zona fasciculata but lacked the cordlike arrangement found in that area. The larger nodules often had necrotic or hemorrhagic centers. Large cytoplasmic vacuoles, probably representing lipid material, were present in most cases. Around the periphery of the nodule cords of cells were seen, and on the outer aspect they were contiguous to the zona glomerulosa (fig. 7). Some nodules were

not uniform throughout, but contained islands of pleomorphic cells in some of which the nuclei were enlarged and sharply outlined by chromatin. The medulla in the adrenal glands containing large adenomas was often compressed to the opposite side of the gland (figs. 6 and 8).

Cortical adenocarcinomas.—Four animals of the group developed lesions diagnosed as cortical adenocarcinomas (fig. 9). These were composed of polygonal cells, which generally resembled those of the zona fasciculata; however, they sometimes showed considerable variation in size and morphology, resembling giant cells, cells with double nuclei, and cells with greatly enlarged single nuclei. Mitotic figures were numerous. The cells were arranged in a lobular pattern, each lobule surrounded by a thin endothelium-like covering which formed the wall of a sinusoid with the adjacent lobule. Red blood cells were frequently seen in these sinusoids. In some areas of the tumors, there was a tendency for the cells to be aligned perpendicularly to the blood-vessel walls (fig. 14). Two of the animals bearing adenocarcinoma had numerous metastases in the liver and lungs, and in one instance a metastasis to bone marrow was seen (figs. 12, 13, and 11).

When transplanted subcutaneously into young Osborne-Mendel rats, the tumors grew rapidly, and, as a general rule, they reproduced the microscopic appearance of the primary tumor (fig. 10). The cell types and organization of the metastases to lungs and liver were also similar to those of the primary tumor.

The adrenal glands of the host bearing the transplanted tumor were quite small when compared to those of similar non-tumor-bearing animals (fig. 16). Microscopic examination of these adrenals showed that most of the loss in size could be attributed to atrophy of the cortex (fig. 15). The zona glomerulosa did not appear to be altered, but the zona fasciculata and zona reticularis were profoundly altered and completely lost their characteristic appearance (fig. 17). Small areas of hemorrhage and necrosis were seen, particularly in the region of the reticularis. The fasciculata cells were no longer arranged in the cords characteristic of the normal gland—the cytoplasm was shrunken, most nuclei were pyknotic, and the medullary cells did not appear to be morphologically altered.

Discussion

Though adenomas of the adrenal cortex or medulla are not uncommon in old rats, no published report of adenocarcinoma of the adrenal cortex in the rat has come to our attention. In a study of spontaneous tumors in wild rats, McCoy (*6*) found no adrenal tumors in 100,000 animals, and Woolley (*7*) found no adrenal tumors in 23,000. This absence of adrenal tumors in wild rats may be related to their age at autopsy. Bullock and Curtis (*8*) found 4 adrenal adenomas in a colony of 2,450 aging laboratory rats—an incidence of 0.2 percent. Curtis *et al.* (*1*) reported only one case of adrenal tumor in 31,868 aging rats of many strains; Ratcliffe (*9*) found 1 cortical adenoma among 468 Wistar rats 1 to 3 years of age—an incidence of about 0.2 percent. Saxton *et al.* (*10*), in a study of the influence of nu-

trition on incidence of spontaneous tumors in Osborne-Mendel rats, reported 5 adrenal adenomas in 498 animals—an over-all incidence of 1 percent. However, he found no adrenal tumors in 200 rats on restricted caloric intake. Thus incidence of spontaneous adrenal tumors in animals on unrestricted caloric intake becomes about 2 percent. Nelson (*11*) working with white rats aged 24 months or older estimated the incidence of adrenal adenomas, both cortical and medullary, at about 3 percent and of cortical adenomas alone at 2 percent.

As opposed to these reports of low incidence (under 5%) of spontaneous adrenocortical tumors in laboratory animals, Yeakel (*2*) reported a high incidence, 51 percent in males and 11 percent in females, of medullary hyperplasia in a series of 37 aging Wistar rats. Stewart (*12*) observed an incidence of spontaneous adrenocortical adenomas and hyperplasias in 54.5 percent of old Osborne-Mendel rats 18 months old or older. Gillman *et al.* (*3*) reported the highest incidence (76% in females and 85.5% in males) of spontaneous pheochromocytoma in a line of Wistar strain rats. They described this adrenal tumor as low-grade carcinoma.

Adrenal tumors have been induced experimentally in rats. Thus Moon *et al.* (*13*) induced medullary hyperplasia and neoplasia in rats with pituitary growth hormone; Martin and Baumann (*14*) induced medullary hypertrophy with chronic thiouracil poisoning; Staemmler (*15*) induced medullary tumors with chronic nicotine poisoning; and Dunning and Curtis (*16*, *17*) induced adrenal adenomas with diethylstilbestrol and with estrone pellets. However, adrenocortical adenocarcinoma was not reported in any of these studies.

Hertz, in discussing Woolley's report (*18*) on experimental tumors of the adrenal cortex, observed, "In Osborne-Mendel rats, early ovariectomy will lead to a benign nodular hyperplasia of the adrenal in 15 percent of the rats, although we have not at any time observed a true adrenal carcinoma."

In view of these observations, an incidence of 9.5 percent of adenocarcinoma and 62 percent of adenoma of the adrenal cortex (a total of 71.5% of adrenocortical lesions) in old Osborne-Mendel rats under study is of interest. The observations of Yeakel (*2*), Stewart (*12*), and Gillman *et al.* (*3*), make it probable that these are spontaneous lesions in old female rats. Why they develop so frequently in old females has not been determined. Environmental conditions may influence these lesions, and the dietary treatment in the present experiment may have modified the time of appearance, the frequency, or the severity of the lesion.

Our observations closely parallel those of Stewart (*12*), whose series also included about 40 Osborne-Mendel rats, 18 months or older, which were predominantly females. The adrenocortical adenomas observed by Stewart (*12*) were indistinguishable from the lesion we have described. However, our incidence of 71.5 percent may be considered as slightly higher than their incidence of 54.5 percent. Our incidence of 9.5 percent of fully developed adenocarcinomas may be slightly higher. Though animals in both series ranged from 18 to 30 months in age, the average

age of our animals was about 2 months younger than Stewart's. Granted that these are small differences and in a small series like this statistically insignificant, still we are inclined to feel that dietary treatment of the animals may have slightly modified the time of occurrence, frequency, and severity of this spontaneous adrenal lesion in these old rats.

Anderson (*19*), in referring to human adrenal tumors, states that functioning tumors with rare exception are unilateral and that the adrenal without tumor is often markedly atrophic. He divides the endocrine disturbances associated with adrenocortical tumors into 3 groups: 1. pseudohermaphroditism, 2. adrenal virilism, and 3. Cushing's syndrome. Four cases of adrenocortical tumor reported here were definitely unilateral, and the contralateral adrenal was atrophic. This tumor when transplanted induced atrophy in the adrenals of the host. This is presumably mediated through the suppression of ACTH by an adrenocortical hormone elaborated by the tumor. We have no direct evidence as yet regarding the hormones secreted by this tumor and their biological activities.

Summary

An adrenocortical adenocarcinoma in the Osborne-Mendel rat has been reported. This tumor was observed in female rats which lived to an age of 18 months or more. An incidence of 62 percent of adrenocortical adenoma and 9.5 percent of adrenocortical adenocarcinoma has been reported in 42 female Osborne-Mendel rats which lived to the age of 18 months or more. These animals had received a low-protein (6 and 8 gm. %) synthetic diet containing 0.06 percent *p*-dimethylaminoazobenzene for 280 days and Purina laboratory chow pellets for the rest of their lives. The adenocarcinoma metastasized to the lung and the liver of the animal in which it originated. It was successfully transplanted in 100 percent of trials to young Osborne-Mendel rats, both male and female, and grew to an average weight of 15 to 20 gm. in 3 to 4 months. It metastasized to the liver and lungs of the host. The tumor caused a reduction in the size and weight of the adrenals of the host. Microscopically the adrenal cortex showed atrophy.

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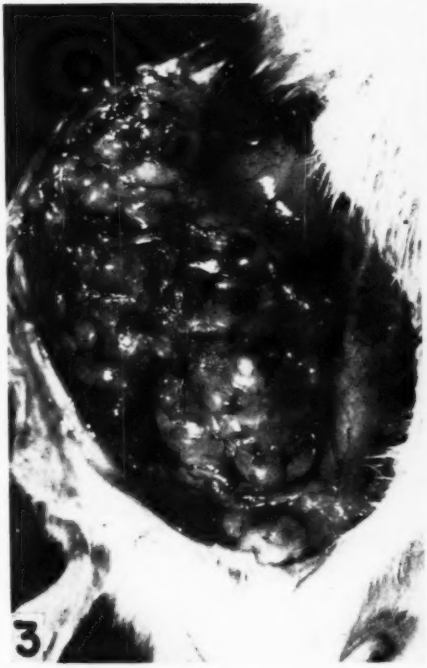
PLATE 58

FIGURES 1 AND 2.—Primary adrenal tumors. × 2

FIGURE 3.—Transplant of adrenal tumor. × 0.9

FIGURE 4.—Metastasis in liver and lung of a rat to which the adenocarcinoma was transplanted. × 1.5



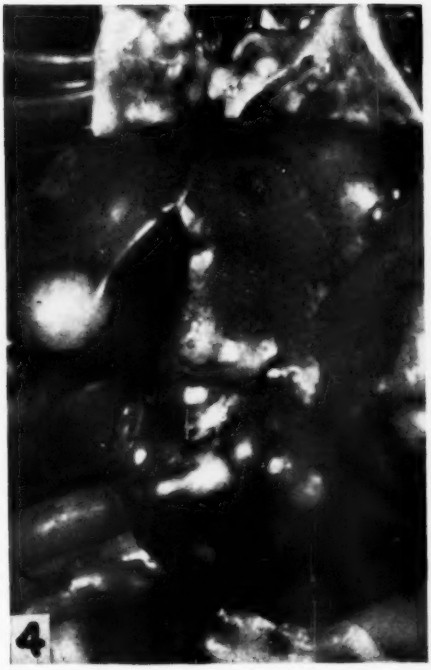


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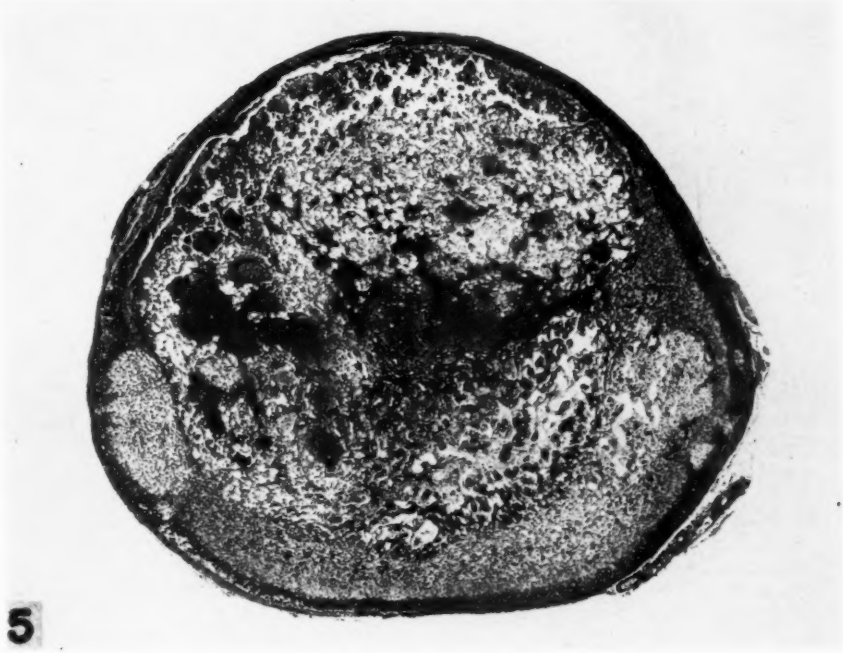
PLATE 59

FIGURE 5.—Adenoma of adrenal cortex in 26-month-old Osborne-Mendel rat. Note central necrosis and hemorrhage, vacuolization of cytoplasm in many of the cells, and displacement of the medulla. Hematoxylin and eosin. × 26

FIGURE 6.—Adenoma of adrenal cortex in a 26-month-old Osborne-Mendel rat. Note islands of tumor cells among the lipid-laden cells. Hematoxylin and eosin. × 25



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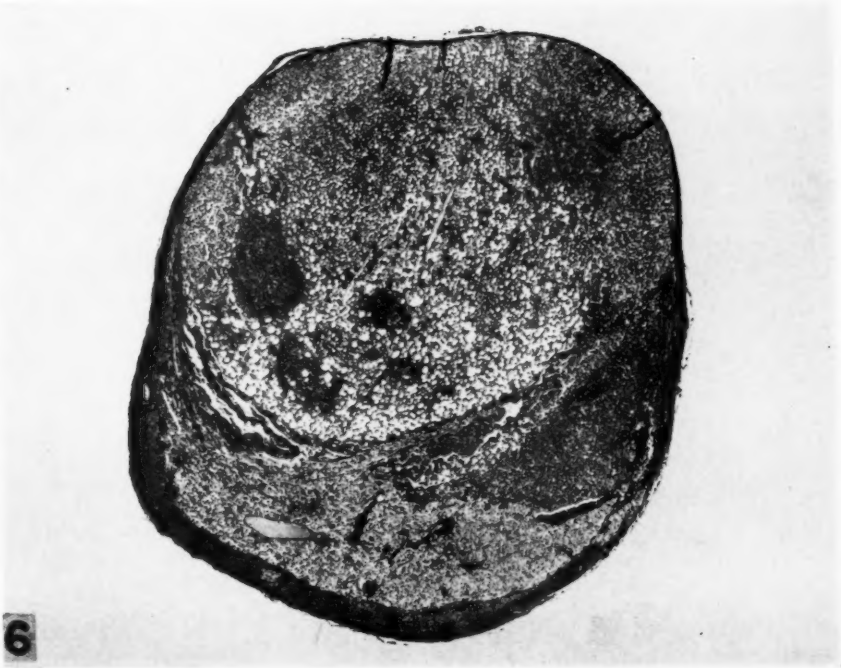

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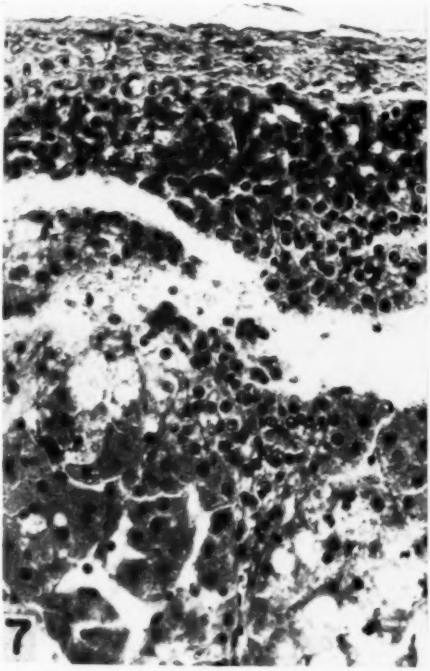
PLATE 60

FIGURE 7.—Higher magnification of periphery of adenoma in figure 5. Note essentially normal zona glomerulosa and capsule. Hematoxylin and eosin. × 260

FIGURE 8.—Higher magnification of cellular island from figure 6. Hematoxylin and eosin. × 260

FIGURE 9.—Microscopic structure of one of the primary adrenocortical adenocarcinomas used in transplantation experiments. Hematoxylin and eosin. × 340

FIGURE 10.—Adrenocortical carcinoma transplant—third generation. Hematoxylin and eosin. × 340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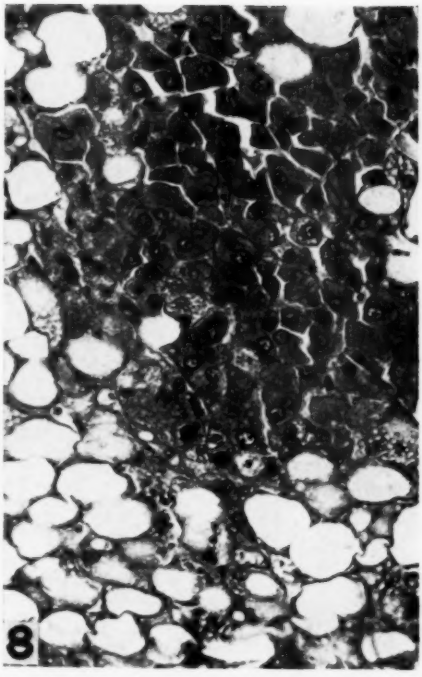
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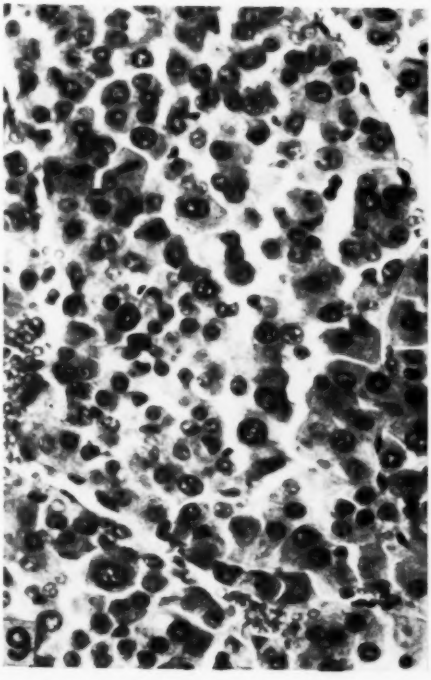
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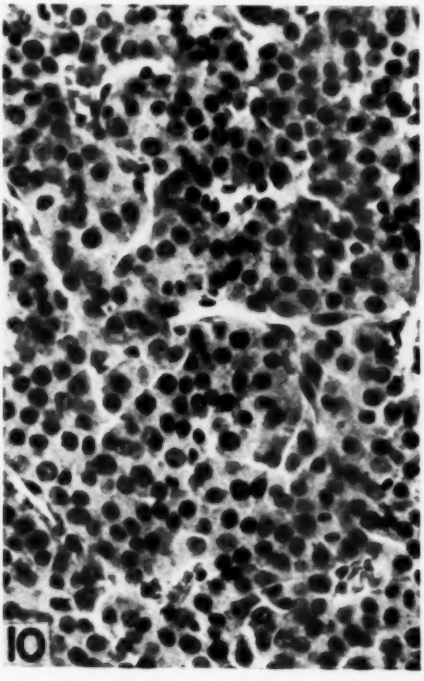
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PLATE 61

FIGURE 11.—Metastasis to bone marrow from a primary adrenocortical carcinoma. Hematoxylin and eosin. × 340

FIGURE 12.—Metastasis to liver from a first generation transplant of adrenocortical carcinoma. Hematoxylin and eosin. × 340

FIGURE 13.—Metastasis to lung from a primary adrenocortical carcinoma. Hematoxylin and eosin. × 200

FIGURE 14.—Adrenocortical carcinoma—second generation transplant showing tumor cells oriented vertically to blood-vessel walls. Hematoxylin and eosin. × 340

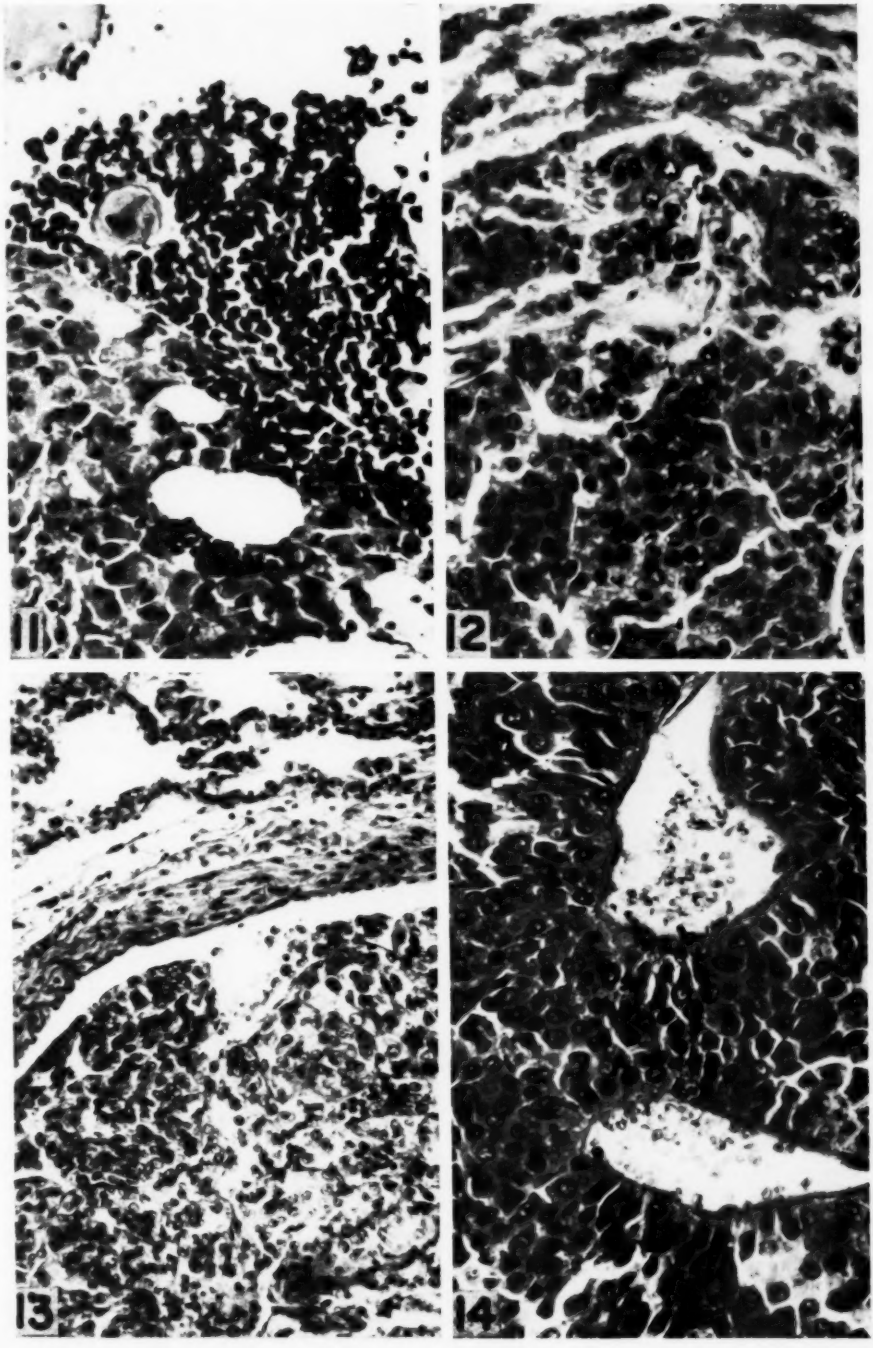
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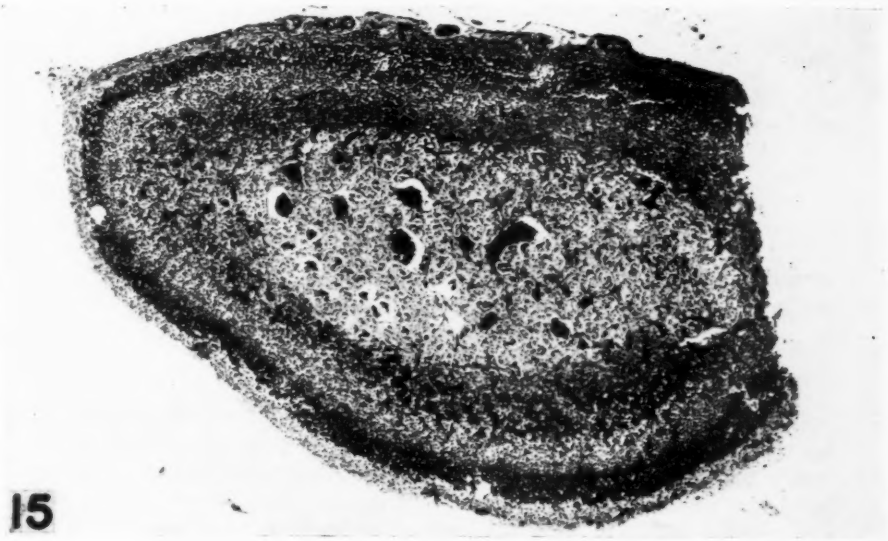
PLATE 62

FIGURE 15.—Adrenal gland of a rat bearing adrenal adenocarcinoma. Note atrophy of the cortex. Hematoxylin and eosin. × 46

FIGURE 16.—Comparison of size of adrenal glands: (A) adrenocortical adenomas in old rats; (B) adrenals of 6- to 11-month-old host rats in which adenocarcinoma transplants are growing; (C) adrenals of male rats 6 to 11 months old; (D) adrenals of female rats 2 years of age or older. × 1.3

FIGURE 17.—Higher magnification of the atrophied adrenal cortex in figure 15 showing disorientation of the cords of zona fasciculata, pyknosis of cells, and congestion in the zona reticularis. Hematoxylin and eosin. × 210



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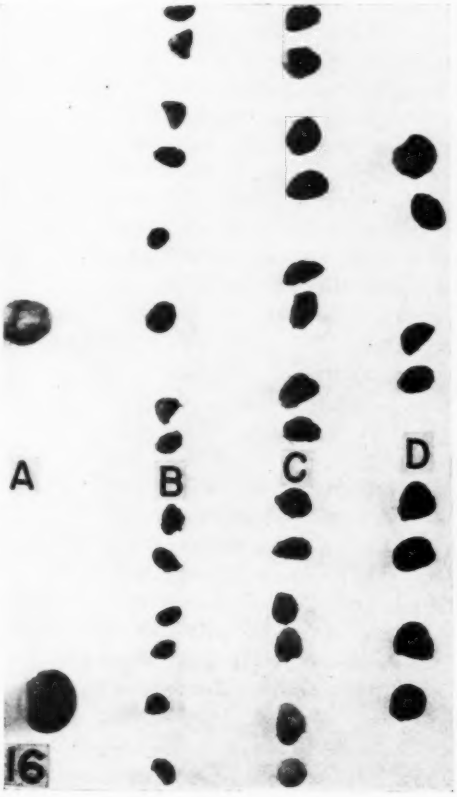

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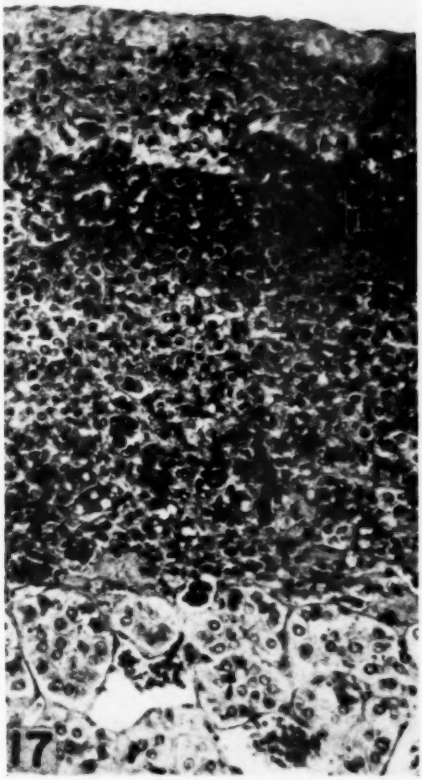

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The Metabolism of Melanomas in Platyfish-Swordtail Hybrids[1]

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Spontaneous tumors in fish resulting from interspecific crossings have been known for several decades. Häussler (*1, 2*) described several melanomas arising in crosses between *Xiphophorus helleri* and *Platypoecilis maculatus* (now *Xiphophorus maculatus*). Gordon (*3–5*) analyzed the inheritance of pigmentation in these two fishes and their hybrids, and showed that the frequency of appearance of the melanoma could be interpreted on the assumption that the occurrence or nonoccurrence of the tumors was under the control of genes.

Further investigation showed that the incidence of the tumor in the hybrid fish was correlated with the presence of macromelanophores in the hybrid, and that the appearance of macromelanophores was controlled by 1 of 5 sex-linked genes in the platyfish.

Reed and Gordon (*6*) described the morphology of the tumors which appeared in the platyfish-swordtail hybrids. In the description of the development of tumor growth which follows, the terminology of pigmented cells which will be used is that agreed upon at the Third Conference on the Biology of Normal and Atypical Pigment Cell Growth (*7*). These terms are as follows:

"Melanoblast: an embryonic cell potentially capable of producing melanin.

"Melanocyte: a mature melanin-producing and melanin-containing cell.

"Macrophage (melanophage): a cell containing phagocytized melanin.

"Melanophore: a pigment effector cell in the lower animals."

In the *Xiphophorin* fishes, the basic melanin pigment of the animal is the result of the presence of two different pigment cells—macromelanophores and micromelanophores. The micromelanophores, uniformly distributed over the adult fish, are small, stellate cells about 100 μ in diameter. The macromelanophores are much larger cells, about 300 μ in diameter, and are usually found in small aggregates, the distribution of which is genetically controlled.

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In their description of the development of the tumor, Reed and Gordon (*6*) divided its course into three phases:

Phase I—Hyperpigmentation: In the first stage of melanoma production, large areas of the fish became darkly pigmented due to the accumulation of numbers of macromelanophores in the corium. These cells were often clumped together in distinct masses during this phase.

Phase II—Invasion and destruction of subcutaneous tissues by the macromelanophores: Consolidation of the groups of macromelanophores was usually accompanied by changes in the tissues covered by the pigmented cells. Destruction of all connective tissues except cartilage occurred quite fast. If the tumor was developing on a fin, the edge of the fin became ragged and the naked fin rays projected from the pigmented tissue. At the same time, the macromelanophores infiltrated the deeper muscle tissue, migrated along the connective tissue separating the muscles, and finally surrounded and destroyed the individual muscle cells. In some cases the fin was completely lost and locomotion seriously affected.

Phase III—Overgrowth: The third stage of the melanosis manifested itself with the appearance of a clublike overgrowth of tumorous tissue. Invasion of the underlying tissues continued, resulting in considerable impairment of activity.

Since the presence of pigment made the identification of cell types in the tumor overgrowth difficult, Grand, Gordon, and Cameron (*8*) analyzed the third stage of melanoma in tissue culture. They were able to show that the hyperplastic material contained melanophages, fibrocytes, melanocytes, and macromelanophores. With the exception of the macromelanophores, which are apparently unique to fish, there was a considerable resemblance between the cells found in fish melanomas, and those found in mouse and human melanomas.

The oxidative metabolism of various mouse and human melanomas has been investigated by several authors. Hogeboom and Adams (*9*), using aqueous extracts of the Harding-Passey mouse melanoma, were able to show that the extracts were active against both tyrosine and dihydroxyphenylalanine (DOPA). They were also able to show by fractionation with ammonium sulfate that the activity against these two substances could be obtained in separate fractions.

Greenstein *et al.* (*10*) and Greenstein and Algire (*11*), utilizing homogenates made from human melanomas, as well as the S91 mouse melanoma, found tyrosinase and DOPA oxidase activity in these tissues. When homogenates of the amelanotic S91A melanoma from the same strain of mice were used, these two enzymes could not be demonstrated.

Burk *et al.* (*12*) measured anaerobic and aerobic glycolysis in a series of tumors including the Harding-Passey and S91 melanomas, and the Algire S91A amelanotic melanoma. These tumors were compared with the Earle L sarcoma and the C3HBA adenocarcinoma (both from mice). They found that with respect to glycolysis, all three of the melanoma strains fell within the range for malignant tumors in general.

In a further analysis of these five tumors, Burk *et al.* using *p*-phenylene-

diamine (PPDA) as a hydrogen donator, tested the activity of the cytochrome-oxidase complex. The three melanomas not only showed a much greater percentage increase than did the Earle sarcoma or the C3HBA adenocarcinoma upon the addition of PPDA, but exceeded the range of stimulation for malignant growths in general. This result was interpreted as an indication that in the melanotic tumor tissue a greater percentage of the cytochrome c existed in the oxidized form.

The enzyme content of the particulate fractions of various melanomas has been investigated by duBuy *et al.* (*13*). They reported that the modified mitochondrial fraction of the Harding-Passey melanoma possessed tyrosinase and DOPA oxidase activity. The S91 melanoma particulate possessed DOPA oxidase activity but little tyrosinase, and the amelanotic melanoma was not active against either tyrosine or DOPA.

In the experiments reported in this paper the oxidative metabolism of various regions of fish hybrid melanomas has been measured. Since certain cells in these tumors are very similar morphologically to cells found in the mouse melanomas, comparisons have been made between the metabolism of fish and mouse melanomas.

It is possible to grow cells of the fish melanoma on the extra-embryonic membranes of the fish embryo. In this way the types of cells found in the overgrowth region of the tumor have been examined, and the metabolism of several regions of the tumor has been analyzed on the basis of the types of cells found in each region.

Materials and Methods[2]

Platyfish, homozygous for the gene for dorsal macromelanophore spotting, were crossed with wild swordtails (*Xiphophorus helleri*). The resulting hybrids, showing hyperpigmentation around the dorsal fin region, were then backcrossed to swordtails. Fifty percent of the backcross offspring showed a macromelanophore hyperpigmentation and produced, almost without exception, "Phase III melanomas" sooner or later in their life span.

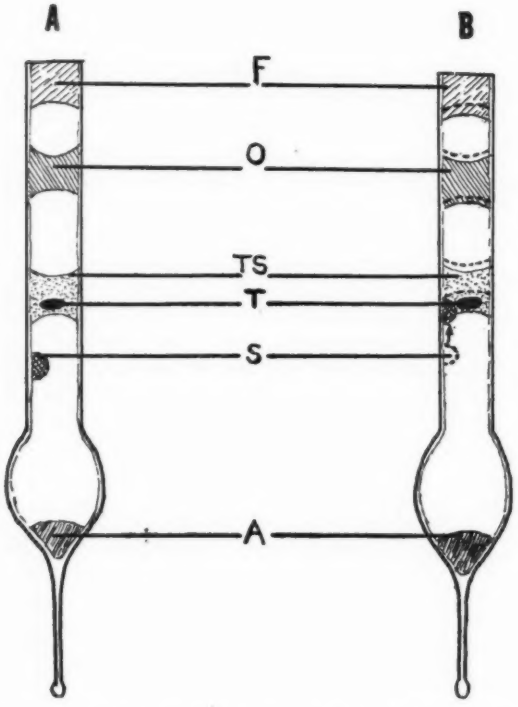
The oxygen-consumption measurements were carried out using the Cartesian diver apparatus as constructed by Boell and Needham (*14*). Since several modifications on the loading technique have been devised, it is perhaps in order to describe the technique briefly.

The tissue to be measured was removed from the fish and divided into small pieces under a dissecting microscope by means of iridectomy scissors. Loading of the divers was carried out by means of micropipettes held in a rack on the wall. These pipettes usually consisted of a piece of thermometer tubing whose inside bore had been calibrated volumetrically. By means of a special rack and pinion, the diver was raised under the appropriate pipette and a measured amount of liquid was forced out

[2] The fish used in these experiments were obtained from the Genetics Laboratory of the New York Zoological Society at the American Museum of Natural History through the courtesy of Dr. Myron Gordon, to whom the authors express their sincere appreciation for his encouragement and advice.

of the pipette and into the diver. Text-figure 1-A illustrates the appearance and distribution of seals. The use of KOH to absorb CO_2 below the tissue seal, as well as the use of an oil seal to prevent osmotic exchange between the fluids of the diver and the flotation medium, are standard procedures.

The introduction of a side drop in the neck of the diver, which permits the addition of substrates to the tissue seal after a period of "endogenous



TEXT-FIGURE 1.—*A*. Seals in a Cartesian diver before addition of substrate. F=flotation medium; O=oil seal; TS=tissue seal; T=tissue; S=substrate side drop; A=alkali.

B. Change in the position of seals after application of pressure. Surface tension causes the incorporation of the substrate drop with the tissue seal. Upon release of pressure the volume of the diver is unchanged.

respiration," is the result of a suggestion by Claff (*15*). This modification makes it possible to carry out all standard Warburg procedures in the divers. In order to prevent the side drop from flowing down the neck, the diver was coated with Desicote prior to use. This holds the small (0.3 $mm.^3$) drop in place on the neck and facilitates loading.

After the diver has received the side drop, it is completely filled with

"tissue seal liquid," usually Niu's solution,[3] the tissue is floated into place down the neck of the diver, the excess seal sucked off, and the neck of the diver wiped dry. In addition to Niu's solution, Krebs-Ringer's solution with and without glucose, and also *M*/15 phosphate buffer have been employed as "tissue seal liquids," without effect on the respiration of the tissues. Introduction of the oil was done in the usual manner, *e.g.*, a micropipette was introduced to the desired level and a measured quantity blown out, producing a complete seal.

The diver is then placed in the bath, balanced, and read for a sufficient number of intervals to establish the slope and linearity of the endogenous respiration. The addition of the substrate to the tissue is accomplished by the cautious application of positive pressure to the diver medium, with the result as seen in text-figure 1-B. As all the seals are pushed down the tube, the tissue seal comes into contact with the side drop (which does not move), and surface tension causes the incorporation of the side drop into the tissue seal. When the pressure is gently released, all the seals return to their original position, the volume of the diver is unchanged, and the substrate is now in contact with the tissue, permitting measurements of oxygen (or any other gas) consumption before and after the addition of a substrate.

The respiration of the tissues was compared on the basis of total (Kjeldahl) nitrogen determinations by modification of a method reported by Boell (*16*), a description of which has been published (*17*).

In the experiments reported in this paper, it was of particular interest to correlate, if possible, the type of oxidative metabolism occurring in a particular region of the tumor and the types of cells found in that portion of the growth. Ordinary histologic methods could not be easily used for these tissues because of the abundance of pigment, and in any event the presence of a particular cell in a section did not indicate that the cell was alive and metabolically active. One possible solution to this problem was the technique of tissue culture which has been applied to these cells by Grand and Cameron (*18*). However, since embryonic transplantation was being carried on in this laboratory for the purpose of investigating other characteristics of the fish melanoma, advantage was taken of the availability of swordtail embryos; and pieces of melanoma taken from the same region of the tumor as those used in the respiration studies were transplanted to the extra-embryonic membranes of the embryo, usually in the region of the mesenteric vein. In this region the grafts attached and grew very well. The tumor cells spread out in a sheet and can be photographed, counted, and even retransplanted; or if need be, they can be removed from the graft and introduced into a diver and tested metabolically.

[3] Niu's solution: Use equal parts of A and B.

A

	gm.
NaCl	3.4
KCl	0.05
$Ca(NO_3)_2.4H_2O$	0.08
$MgSO_4$	0.10
Distilled water	500 cc.

B

	gm.
Na_2HPO_4	0.11
KH_2PO_4	0.02
Distilled water	500 cc.

Further separation of the cell types was possible in the types of melanoma used by taking advantage of certain migratory characteristics of the tumor cells. In the earliest phase of the tumor growth, the macromelanophores are restricted to the corium. As the growth progresses, there is a distinct tendency for the macromelanophores to invade the deeper tissues of the back. They migrate along the connective tissues, isolate, and eventually destroy the muscle bundles. It is possible to obtain these invasive macromelanophores free from other pigmented cell types, although a certain amount of connective tissue and small amounts of muscle were sometimes included with these cells. These invasive cells will be referred to subsequently as "deep melanoma."

Results

Metabolic studies have been carried out on cells obtained from the three phases of tumor growth described in the introduction. In all cases the tissues were taken from young adult hybrid fish resulting from the mating of a wild swordtail to a spotted platyfish-swordtail hybrid.

As a source of cells in Phase I (hyperpigmentation) of tumor development, an area of skin was selected which was abundantly supplied with macromelanophores, but which appeared to be free of melanoma. The scales were removed and the underlying skin with its attached pigment cells was carefully dissected free. The pigment cells in this phase of melanoma development, although much more abundant than the macromelanophores in a normal fish, were not significantly different in morphology from the normal pigment cells. Figures 1 and 2 show the similarity between these two groups of cells.

The Phase II (invasive) and Phase III (overgrowth) tissues were obtained from 14 fish bearing Phase III melanomas. In order to obtain both types of tissue, the tumor was divided into three regions—superficial, middle, and deep—and all were grafted to the extra-embryonic membranes of fish embryos.

Superficial Region of the Melanoma

The superficial region consisted of the epithelium covering the tumor and the relatively few pigment cells clinging to it. In general the pigmented cells of this region consisted of three types:

1. Small, roughly triangular cells, very heavily filled with pigment, which were probably melanophages.
2. Somewhat lighter pigment cells which appeared very similar to the normal micromelanophore population of the epidermal layer.
3. A variable population of middle-melanoma-type cells (see below) which adhered to the epithelium as it was removed.

Since the cell population of this region was quite variable because of unavoidable contamination with cells typical of the middle region of the melanoma, although the grafts were successful results obtained from this portion of the tumor were variable; after preliminary determinations, this

region was dissected away from the middle-melanoma overgrowth and not analyzed further.

Middle Region of the Melanoma

The second division represented the main body of the tumor and was very characteristic and easily distinguished from either the superficial or deep regions. This tissue was the overgrowth characteristic of Phase III. It was a lighter brown and had a very soft, almost powdery, texture. When cut, large numbers of pigment granules were released into the medium. When pieces of this portion of the tumor were transplanted into normal swordtail embryos, as a method of analyzing the cell types contained in the tissue, at least four types of cells appeared in the outgrowth from the graft. Figure 3 shows the result of such a graft. In this case, a small piece (0.1 mm. on a side) of tissue from a middle melanoma was placed under the serosa of a Stage 22-Tavolga (*19*) swordtail embryo. Outgrowth began in about 24 hours and continued actively for periods up to 3 weeks. Figure 4 shows the same graft after it was removed from the host. At this magnification it was possible to distinguish several types of cells in the middle-melanoma tissue:

1. The first cells to appear were usually the melanophages, which have been described as part of the cellular component of the superficial tissue.

2–3. Shortly after the melanophages emerged, two types of melanocytes appeared. The first of these consisted of long, highly branched, very darkly pigmented cells. The second type was a large platelike cell which was very lightly pigmented. On the basis of its size (300 μ) the latter cells may represent a stage in the development of a macromelanophore-type cell as described by Marcus and Gordon (*20*). Each occurred in considerable numbers, with the long, thin cell being much more abundant.

4. After the graft had been in place for several days, occasional pigment cells appeared which were similar to the macromelanophores of the normal skin.

Deep Region of the Melanoma

What will be referred to as the deep portion of the melanoma was made up of macromelanophores which had invaded normal tissue under the body of the tumor proper. These cells corresponded to the invasive melanophores of Phase II. They were very large, very dark, highly branched cells which apparently migrated along the connective tissues in the muscles of the back and could be obtained in considerable quantity, by microdissection, free of any other type of pigment cell. Although a number of grafts have been made, the deep-melanoma cells do not migrate readily in the extra-embryonic membranes of a fish embryo.

As can be seen from table 1, the oxygen consumption in the absence of added substrate (endogenous respiration) of the middle melanoma is ap-

TABLE 1.—*Oxidative enzyme activities of various portions of fish melanoma—data expressed as* $Q_{O_2}(N)$

Tissue	Endogenous respiration	Substance added*					
		0.02 *M* PPDA	1.4×10^{-7} *M*† cytochrome c	Cytochrome c† + PPDA	0.5 *M* succinic acid† + cytochrome c	0.4×10^{-3} *M*† tyrosine	1.0×10^{-3} *M*† DOPA
Hyperpigmented cells							
Average:	0.26						
Predump		0.37	0.26	3.0 μl./hr.‡	0.31	0.18	0.12
Postdump		0.51	0.31	4.0 μl./hr.	0.55	0.39	0.26
Change (%)		*38%*	*20%*	*33%*	*83%*	*107%*	*117%*
Number of determinations	5	2	1	1	2	2	3
Number of animals–4							
Deep-melanoma							
Average:	0.29						
Predump		0.5	0.14	0.25	0.25	0.17	0.47
Postdump		2.6	0.14	1.10	1.21	0.41	2.00
Change (%)		*430%*	*0%*	*340%*	*386%*	*141%*	*408%*
Number of determinations	10	2	1	2	2	5	2
Number of animals–14							
		Dump ascorbic acid		Cytochrome c + ascorbic acid			
Middle-melanoma							
Average:	1.05						
Predump		1.02	0.80	1.46	1.06	1.08	1.20
Postdump		1.76	0.94	2.70	1.52	1.78	1.60
Change (%)		*71%*	*17%*	*58%*	*43%*	*62%*	*30%*
Number of determinations	10	2	2	2	4	6	3
Number of animals–14							

*Each diver carried the following seals: oil—1.0 mm.³, tissue-seal liquid—1.5 mm.³, side drop—0.3 mm.³, alkali—1.0 mm.³.

†Tissue-seal liquids for determination of:

a) Succinoxidase activity after Umbreit (*22*): 1.0 ml.–0.1 *M* phosphate buffer—*p*H 6.8; 0.4 ml.–1.4×10^{-7} *M* cytochrome c; 0.3 ml.–4×10^{-3} *M* $CaCl_2$; 0.3 ml.–4×10^{-3} *M* $AlCl_3$.

b) Cytochrome-oxidase activity after Umbreit (*22*): 1.0 ml.–0.1 *M* phosphate buffer—*p*H 6.8; 1.0 ml.–1.4×10^{-7} cytochrome c; 0.3 ml.–4×10^{-3} *M* $AlCl_3$.

c) Tyrosinase and DOPA oxidase activity: Niu's solution—*p*H 6.9.

‡Since the nitrogen analyses were lost on this determination the results are expressed as μl. of oxygen consumed per hour.

proximately four times as great as that of either the deep-melanoma (Phase II) or the hyperpigmented (Phase I) cells. Preliminary experiments on the middle-melanoma tissue indicate that a portion of this oxygen consumption amounting to 50 to 60 percent can be inhibited by potassium cyanide, used according to the method of Robbie (*21*). The addition of 10^{-2} and $10^{-3}M$ diethyl-dithiocarbamate (DDC) resulted in a decrease in oxygen consumption of about 35 percent. The latter evidence suggests that a considerable portion of the respiration observed in the middle-mel-

anoma cells was copper catalyzed, but the possibility should not be overlooked that at the concentration used, DDC might bind iron as well as copper.

Of some interest is the observation that under the conditions of the cyanide experiment a portion of the oxygen consumption amounting to some 40 to 50 percent of the total was not inhibited. This suggested that a system, not catalyzed by heavy metal ions, might be involved in a considerable proportion of the oxygen consumption of these cells.

The possibility should not be overlooked that the failure of cyanide to inhibit the endogenous respiration may be due to artefacts caused by the manipulation of the tissues. Greenstein (*10*, *11*) and subsequently Riley (*23*) were able to show the presence of such an artefactual cyanide-resistant system with DOPA and PPDA in melanoma homogenates.

The extent of cell damage in the tissues used in these experiments is of course somewhat greater than that experienced in more gross methods, since the small size of the pieces exposes a relatively greater surface area than is the case in tissue slices. This objection is more pertinent in the case of the deep-melanoma region than in the middle region. Middle-region and hyperpigmented cells are obtained with a minimum of dissection and disturbance, while deep cells must be dissected free from the surrounding connective tissue and are therefore subjected to more trauma. Since PPDA is not used in the cyanide experiments on middle-region cells, it seems unlikely that the same system as described by Riley is active in the middle-melanoma pieces. A further argument against the artefactual nature of the cyanide-resistant respiration may be found in the fact that upon the addition of PPDA to the tissues there is no large increase in oxygen consumption by the middle-melanoma-region cells.

No significant difference existed between the endogenous-oxygen consumption of the macromelanophores of the hyperpigmented area and that of the deep-melanoma macromelanophores. Both groups of cells showed a very low respiratory rate. Although it is not indicated in the table, it has been our frequent experience while measuring the oxygen consumption of deep tissue to have the endogenous-respiration period show a slight positive value. However, the tendency was usually very slight, and, usually, just over the error of the method. No information has been obtained as to what gas might be evolved from such a tissue, although carbon dioxide may be safely eliminated as a possibility under the conditions of the experiment.

The data in the second column of table 1 are a measure of the degree to which the cytochrome oxidase and associated cytochrome c systems are saturated *in vivo*. Since cytochrome c is usually considered to be the limiting component in this system, the addition of a hydrogen donor, such as PPDA or ascorbic acid, in excess, should give a relative measure of the amount of cytochrome c available for the oxidation of the added substrate.

Column 3 of the table can be used to give a relative measurement of the amount of available substrate in the presence of an excess of cyto-

chrome c and serves as a check on the second column. It is of interest to note that the macromelanophores, regardless of their position in the tumor, seem to contain little substrate which is not being adequately oxidized by the cytochrome c already present in the cells. Thus the addition of more cytochrome c does not increase the oxygen consumption in any of the tissues studied.

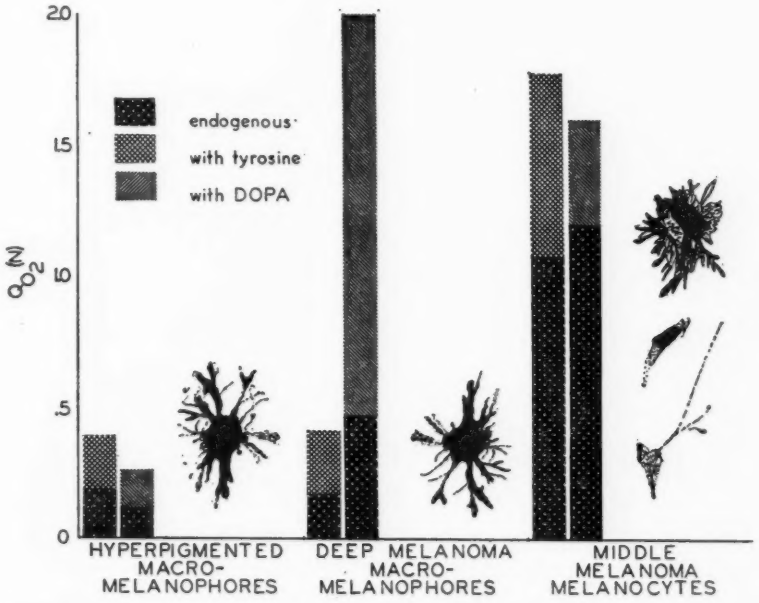
Tumor tissues have, in general, proved to have a relatively poor reserve capacity with respect to the cytochrome system. The range of response of various tumor tissues as reported by Burk (*12*) was from 0 to 120 percent of the endogenous respiration. Our figures for middle melanoma and hyperpigmented epithelium fall into this class. However, the results obtained with the deep-melanoma tissue bear mentioning at this point. As can be seen from the table, the oxygen consumption of the deep tissue after the addition of PPDA was increased by 430 percent. This increase is borne out by a similar increase when PPDA and cytochrome c are both added to the tissue. This is in agreement with Burk, who found that the addition of excess PPDA increased the oxygen consumption of the Harding-Passey and S91 melanoma slices up to 700 percent of the endogenous respiration.

In column 4 of the table, the system is supplied with cytochrome c and ascorbic acid (or PPDA) in excess and represents a relative measure of the activity of cytochrome oxidase. Accordingly, the rate of oxygen consumption should be determined by the activity of this enzyme. No significant differences exist, on a percentage basis, between the hyperpigmentation macromelanophores and the middle-melanoma melanocytes with respect to their activities against cytochrome oxidase. As has been mentioned before, the difference between these tissues and the deep-melanoma cells is very marked indeed.

The column in the table dealing with the oxidation of succinic acid serves a dual purpose. Succinic acid in the presence of succinoxidase can serve as a much more natural hydrogen donor than either PPDA or ascorbic acid. In addition to indicating the activity of succinoxidase in the cells of the melanoma, the activity of the tissue after the addition of this substance, in the presence of excess cytochrome c, serves as a check on the PPDA determination of the activity of cytochrome oxidase. The tremendous activity of the deep-melanoma cells, upon the addition of succinic acid, verifies the observations made above with PPDA and suggests that these deep cells possess a potentially very active aerobic transport system, which lacks substrate for its activity. It should be noted, however, that the respiration of the deep tissue in the presence of succinic acid is in the same range as that of the middle-melanoma cells in the presence of succinic acid. This would suggest that the difference in the endogenous respiration of the deep and middle melanoma may be merely the result of differential availability of substrate.

The relationship between the types of cells found in the various regions of the melanoma and their activity against tyrosine and DOPA, is seen in text-figure 2. Considering first, the activity of the three tissues

against tyrosine, it can be seen that all possess an active tyrosinase. The activity of the hyperpigmentation cells and the deep-melanoma macromelanophores against excess tyrosine is about equal, whereas the activity of the middle-melanoma melanocytes is almost three times as great. In these experiments the actual activity of tyrosinase is not measured, since from the data it was not possible to determine how much of the endogenous respiration of the middle cells was due to the activity of tyrosinase or DOPA oxidase acting upon normally present substrate. As has already been mentioned, preliminary experiments suggest that a considerable portion of the middle-melanoma respiration may be due to tyrosinase activity.



TEXT-FIGURE 2.—The oxygen consumption of three phases of melanoma development with diagrams of the types of cells found in each phase.

The experiments using DOPA as a substrate yielded very interesting results. The tremendous activity against this substance by the deep-melanoma cells indicates a fundamental difference between these cells and the middle-melanoma and hyperpigmentation cells. The very low endogenous respiration of the deep-melanoma cells in the presence of such an active enzyme argues strongly for a lack of available substrate.

Discussion

The relationship between normal macromelanophores and the Phase I hyperpigmentation macromelanophores has been studied by Gordon (*4*). He was able to show that the presence of normal macromelanophores in

the hybrid was an obligate prerequisite for the formation of succeeding stages in the hyperpigmentation phenomenon. In the same manner, since the presence of superficial melanophores must, according to Gordon's studies, precede and presumably lead to the appearance of the invasive macromelanophores, a serial relationship appears to exist between normal macromelanophore pigmentation, the Phase I hyperpigmentation, and the appearance of the deep, invasive macromelanophores typical of Phase II in the development of the tumor.

The morphologic and genetic criteria which have been used to relate normal fish macromelanophores to the cells of the hyperpigmentation phase and to the invasive cells of the tumor are summarized in table 2. The similarity in size and shape of the normal, hyperpigmented, and invasive cells argues strongly for a relationship between them.

TABLE 2.—*Summary of the genetic and morphologic criteria used to relate the normal macromelanophore to the melanophores of the fish melanoma*

	Normal pigment cell	Hyperpigmented cell	Deep melanophore	Middle-melanoma melanocyte
Size	300μ	300μ	300μ	100μ
Shape	Stellate	Stellate to irregular.	Stellate to irregular.	Fine-branched, fibrous.
Color	Black	Black or gray	Black	Black
Tissue layer	Corium	Corium	Deep connective tissue.	Tumor
Requirements for appearance.	*Sd* gene	Normal macromelanophores.	Normal or hypermelanophores.	Deep melanophores.

The origin of the middle-melanoma melanocytes has not been worked out in detail. These cells may first be seen in areas of hyperpigmentation just before the actual appearance of the typical Phase III overgrowth. Whether or not these cells are derived from the macromelanophores cannot be determined cytologically at this time. The small, heavily pigmented melanocytes which make up the greater portion of the overgrowth are unique to this region of the tumor and do not resemble the macromelanophores except in the possession of melanin. Since the melanocyte is the predominant cell type of the middle melanoma, it would seem likely that the metabolism of this region is that of this cell type. A comparison of the metabolism of the cell types found in the three regions of the melanoma is presented in table 3.

In considering the relationship of the cells in table 3, it should be pointed out that perhaps the single difference between the metabolism of the deep-melanoma cells and the middle-melanoma cells is the presence in the latter of a substrate capable of oxidation. Thus, the capacity for rapid oxidation exists in the deep cells but is not manifested due to the absence of a substrate. This accounts for the low endogenous respiration. Although morphologically these two cell types are very different, metabolically they are very similar indeed, and the possibility exists that they are causally related.

That the hyperpigmented cell and the deep-melanoma cell are related to each other is shown by the genetic studies. However, these two cells are different in their potential oxidative capacity, and are therefore, metabolically, quite different.

It follows from the above considerations that the two morphologically similar cells are metabolically different, while the two morphologically different cells are metabolically very similar.

The question arises as to the source of substrate being utilized by the melanocytes. Since the appearance of abundant melanocytes is in every case preceded by the invasive destruction of the underlying tissues by the deep melanophores, it is possible that the very destruction of the connective tissue and muscles by the deep-melanoma cells may be producing the material upon which the melanocytes depend for subsistence. From a metabolic point of view the melanocytes' ability to obtain substrate from the cellular debris may be the distinguishing difference between these cells and the deep macromelanophores. It is not impossible that the presence of breakdown products acting upon the adaptive

TABLE 3.—*Summary of the metabolic data which may be used to suggest a relationship between the deep-pigment cell and the middle melanocyte*

	Hyper-pigmented cell	Deep-melanoma cell	Melano-cytes
Endogenous respiration	Low	Low	High
Response to added PPDA or succinate	Low	High	High
Capacity to utilize endogenous substrates	Low	Low	High

mechanisms of the deep melanophores might result in the production of melanocytes.

Certain assumptions are implicit in the design of the experiments summarized in the first four columns of table 1. It has usually been found in measurements of cytochrome-oxidase activity that the activity of this transferring enzyme exceeds the maximum rate of transfer of any one of the component dehydrogenases from which it normally receives electrons. It has been further found that the concentration of cytochrome c frequently imposes a limitation upon the rate of electron transfer, and, accordingly, dehydrogenase activity is measured in the presence of excess cytochrome c. A third assumption that is considered to be valid for most cells is, namely, that the principal mechanism for the oxidative removal of hydrogen from any substrate takes place through the cytochrome system.

If cytochrome c acts as a limiting substance on the rate of hydrogen transfer, it is to be expected that the addition of cytochrome c, which increases the carrying capacity of the cytochrome system, would result in an increase in oxygen consumption. As a check on this point, if cytochrome c is limiting (assuming that all, or nearly all, of the cell cytochrome c is already occupied with the transfer of hydrogens from the normally

occurring substrates), the introduction of additional substrate would have little effect on the activity of the cells.

It can be seen from the results obtained with the deep-melanoma cells that neither of these anticipated results has been realized. The results obtained show that the addition of cytochrome c has no effect on the oxygen consumption of the cells and that the addition of excess substrate in the absence of excess cytochrome c results in relatively enormous increases in oxygen consumption.

These results may be interpreted in three ways. The assumption that in these cells there is an abundance of cytochrome c and a very great dearth of substrate upon which the cellular enzymes may act would explain the observed phenomena. Addition of substrate, under these circumstances, would result in a large increase in the observed oxygen consumption, inasmuch as the lack of substrate was limiting the rate of metabolism. Likewise, the addition of cytochrome c would have no effect in the absence of substrate, since under these circumstances it would serve only to increase the cytochrome c already in excess in the cells. This situation is not typical of cells in general, but these are atypical cells.

Another possible interpretation is that the more labile intermediate metabolism has been damaged in some way, so that endogenous sources of substrate (proteins, amino acids) are not being utilized at anything like the potential rate (as for example in middle-region cells). Under these conditions addition of cytochrome c would have no effect. However, since cytochrome oxidase and succinoxidase are relatively "tough" systems, large increases in oxygen consumption would be expected when PPDA or succinate are added. In these cases, cytochrome c might well give an additional response. It seems unlikely that the damage to intermediate metabolic systems could have come from manipulation, although the possibility should not be overlooked that in the deep-region cells these enzymes may be more fragile than in the middle regions of the melanoma, since the cell types in the two regions are different.

An additional possibility arises from the knowledge that tumor tissues in general are relatively low in activity with respect to the cytochrome system. It may be possible that the hydrogen from PPDA and ascorbic acid is being transferred to oxygen by another route entirely and that the addition of excess cytochrome c has no effect on the oxygen consumption because the cytochrome system does not play a predominant role in these cells.

The presence of an active tyrosinase in these cells suggests the possibility that the transfer of hydrogen in these deep-melanoma cells may take place with the intervention of this enzyme. Although Boswell and Whiting (*24*) and Nelson and Dawson (*25*) have postulated that in the potato this enzyme may serve a respiratory function through the enzymatic oxidation of DOPA to DOPA quinone and back, Schade *et al.* (*26–28*) have shown that the terminal electron transfer system in exhaustively washed potato slices was the cytochrome system. However, if the tyrosinase system were active in these cells and were responsible for the oxidative activity

found upon the addition of PPDA and ascorbic acid, it could be expected that the rate of oxidation in the presence of these substrates would be independent of cytochrome c. This condition is borne out by the data. Riley, Hobby, and Burk (*23*) were able to show that a synergism existed between PPDA and DOPA. Accordingly, the addition of PPDA to a system already containing DOPA would bring into action a hydrogen-transferring mechanism of considerable capacity. It could also be expected that DOPA, which represents a special hydrogen donor for this system, would contribute hydrogens to the system at just about the same rate as they would be contributed by other more generalized substrates. This is indicated by the data on DOPA oxidation, namely, that DOPA is oxidized by these cells at a rate which is comparable to the rate of oxidation of PPDA and ascorbic acid. In addition, the similar rate of oxidation in the case of succinic acid may suggest that in these cells the measurable activity of the succinoxidase is determined by the activity of the hydrogen-transferring systems.

The transfer system postulated in the paragraph above requires for its action the presence of some hydrogen acceptor present in considerable quantity. If this assumption is correct, it is difficult to understand how the basal respiration of this group of cells could be so low. At this point an apparent variation in the data for the deep-melanoma cells might be discussed, since it has bearing on this point. It can be noticed that while the percentage stimulation of the tissue after the addition of PPDA, DOPA, and PPDA and cytochrome c is quite similar, the actual amount of oxygen consumed by the cells varies considerably, and that the amount of stimulation depends upon the prestimulation respiration. In other words, if the basal respiration of the cells is high, then the oxygen consumed after the addition of any of the substrates will be large, whereas, when the pre-addition oxygen consumption is low, there will be little post-addition respiration. This suggests that the pre-addition oxygen consumption reflects the concentration of some substance in the cells which, upon the addition of a hydrogen donor, becomes active in the transport of hydrogen.

If the above assumption is correct, apparently the hydrogen acceptor is present in the cell. We are no closer to knowing what the transfer agent may be, nor do we have any definite knowledge as to what systems it requires for its activity, but its concentration apparently controls both the pre- and post-addition oxygen consumption in these cells.

According to this assumption, therefore, the metabolic situation in the deep-melanoma cells appears to be that the cells have developed a hydrogen-transporting system of a high degree of efficiency; but under the conditions in which they find themselves, there is little available substrate, and accordingly their respiratory rate is low.

Summary

The oxidative metabolism of three phases of the platyfish-swordtail hybrid melanoma has been measured with the aid of the Cartesian Diver Ultra-micro-respirometer. The premalignant pigment cells of the hyper-

pigmented phase of tumor development were found to possess a low endogenous respiration and a low respiratory capacity, as measured by the addition of PPDA and excess cytochrome c. Against succinic acid, tyrosine, and DOPA, their proportional activity was in the range for normal tissues.

The tissues from two regions of the tumor were examined, and it was found that the tissues from the middle of the melanoma, consisting of darkly pigmented melanocytes, showed a high endogenous respiration and a fairly low respiratory reserve capacity. The tumor tissues lying deep in the tissues of the host, and believed to be the causative cells of the tumor, showed a very marked response to the addition of PPDA and cytochrome c. Since the addition of cytochrome c alone did not increase respiration, it was concluded that these cells possessed a large respiratory reserve capacity and no available substrate and that an enzyme system other than the cytochrome system might be involved in the transfer of hydrogen in these cells. It was suggested that a possible system might be tyrosinase acting on DOPA quinone and DOPA.

Comparisons were made among the cells in various stages of tumor development in an attempt to determine whether or not a causal relationship existed between the deep-melanoma cells and the middle-melanoma melanocytes. It was shown that the two groups of cells were closely related metabolically, and it was considered that this might be evidence of their relationship to each other.

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PLATE 63

FIGURE 1.—Photograph of a wild-type swordtail, showing the rarely occurring macromelanophores and the much smaller micromelanophores. × 50

FIGURE 2.—Photograph of a scale removed from a hyperpigmented platyfish-swordtail hybrid. Note the similarity to cells in figure 2. × 50

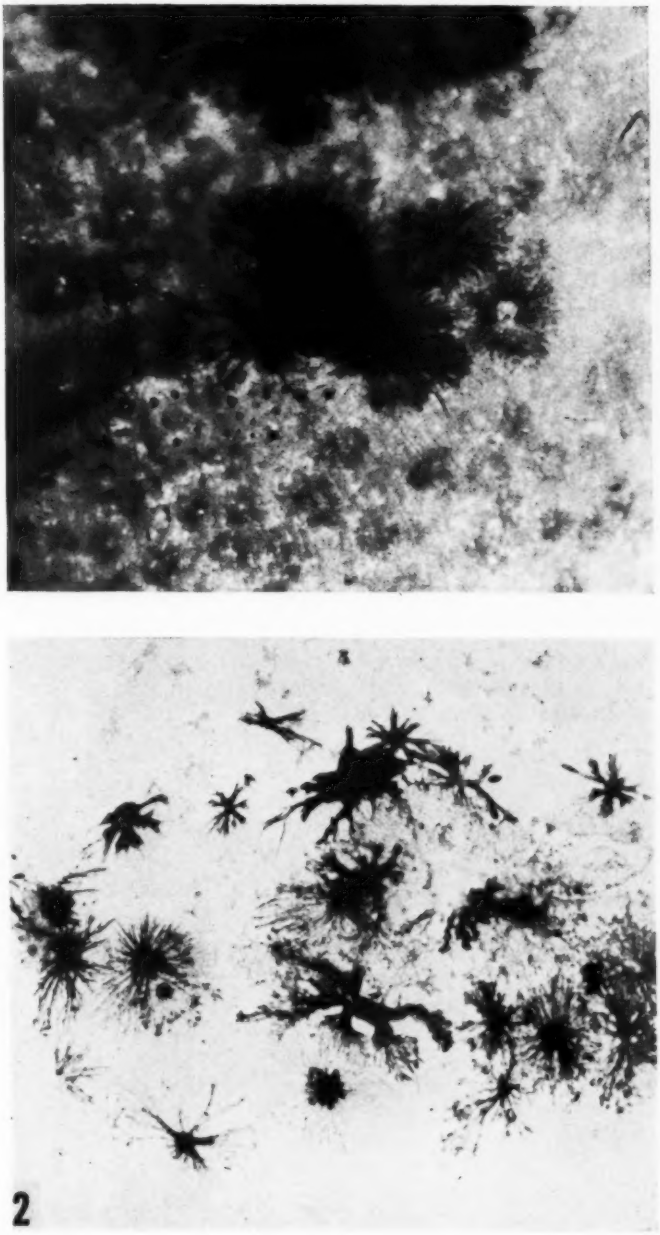

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PLATE 64

FIGURE 3.—Middle melanoma grafted to the extra-embryonic membranes of a swordtail embryo. Note the radial outgrowth of cells from the graft and the melanophages (1) which have migrated from the graft area to the side of the embryo. × 50

FIGURE 4.—A portion of the outgrowth of figure 3 removed from the embryo and photographed to show cell types. 1.—melanophage; 2.—gray melanocyte; 3.—black melanocyte. × 400

1

3.
1
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Development of Pulmonary Tumors in Mice Segregated With Respect to The Three Genes: Dominant Spotting, Caracul, and Fused [1]

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The relationship between a number of known genes and susceptibility to pulmonary tumors has been demonstrated. Lethal yellow (A^y) has been shown to increase susceptibility to both spontaneous pulmonary tumors and those induced with 20-methylcholanthrene (*1*, *2*). In other tests for linkage, flexed tail (*f*) has been associated with decreased susceptibility to spontaneous pulmonary tumors and those induced with 20-methylcholanthrene, and shaker-2 (*sh-2*) and waved-2 (*wa-2*) have been associated with decreased susceptibility to the spontaneous tumors, those induced by 20-methylcholanthrene, and those induced by urethan (*3–5*). Hairless (*hr*) has been associated with a decrease in susceptibility to the spontaneous tumors and those induced by 1,2,5,6-dibenzanthracene (*6*, *7*), and vestigial tail (*vt*), with a decrease in susceptibility to pulmonary tumors induced by 1,2,5,6-dibenzanthracene (*8*). The present experiment was a test for an association between pulmonary tumors induced with 1,2,5,6-dibenzanthracene and the dominant genes—dominant spotting (*W*), caracul (*Ca*), and fused (*Fu*).

Materials and Methods

Mice of strain WCaFu, which carry the three genes—dominant spotting (*W*), caracul (*Ca*), and fused (*Fu*)—were obtained in June 1951 from Dr. Clara J. Lynch of the Rockefeller Institute for Medical Research. These mice were crossed with high-pulmonary-tumor-strain A from the colony maintained by the authors at the National Cancer Institute. The strain A mice are homozygous for the recessive alleles of each of these genes (*w/w ca/ca fu/fu*). Since some of the strain WCaFu mice were heterozygous for 1 or more of these 3 genes segregation was observed in the F_1 animals. The F_1 hybrids heterozygous for all 3 genes, *W/w Ca/ca Fu/fu*, together with a few heterozygous for 1 or 2 genes (*W/w ca/ca fu/fu; w/w ca/ca Fu/fu;* and *w/w Ca/ca Fu/fu*) were then outcrossed to low-tumor-strain 1194 which was homozygous for the recessive allele of each of these genes, like strain A. Strain 1194 has been observed to

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

have a pulmonary-tumor incidence of 4.6 percent in mice of all ages and of 17 percent in mice between 2½ and 3 years of age (*9*). This strain was also obtained from Dr. Lynch in June 1951 and had been inbred for 51 generations at that time. Occurrence of tumors in the offspring of this cross would provide a test for true linkage between any dominant genes affecting susceptibility to pulmonary tumors and the 3 segregating genes and would also provide a test for any effect of these 3 genes themselves upon susceptibility to pulmonary tumors.

A total of 256 [(WCaFu × A)F_1 × 1194] and [(A × WCaFu)F_1 × 1194] mice (129 females and 127 males) was produced for this experiment. In the final data, 17 animals (10 females and 7 males) were omitted because of the failure to obtain an autopsy. The animals were injected intraperitoneally at 2 months of age with 0.5 mg. of 1,2,5,6-dibenzanthracene dispersed in 0.5 cc. of horse serum. They were housed in plastic cages, 8 animals to a cage, and were constantly supplied with Derwood food pellets and tap water. The animals were weighed at weekly intervals beginning at 4 weeks of age. Autopsies were performed at 10 months of age. Nodules on the surface of the lungs were counted with the aid of a dissecting microscope and recorded.

Results and Discussion

In the various segregant groups, susceptibility to induction of pulmonary tumors by intraperitoneal injection of 1,2,5,6-dibenzanthracene was measured by tumor incidence rather than by average number of nodules per individual as was done in other experiments in which tumors occurred in all animals. The nodules were counted in those animals where tumors occurred, but because of the wide fluctuation in numbers and the fact that the incidence in each group fell in the vicinity of 50 percent, it was considered better to make comparisons between the groups on the basis of incidence rather than average number of nodules. The incidence in the groups segregated in respect to each gene pair is shown in table 1. There were 217 progeny of *W/w* F_1 mice, of which 110 were *W/w* and 107 were

TABLE 1.—*Occurrence of pulmonary tumors following intraperitoneal injection of 1,2,5,6-dibenzanthracene*

Genotype	Number of animals	Number with pulmonary tumors	Percent pulmonary tumors	Significance of difference
W/w (dominant spotting)	110	75	68.1	*P* is between 0.5 and 0.7
w/w (nonspotting)	107	69	64.5	
Ca/ca (caracul)	50	31	62.0	*P* is between 0.8 and 0.9
ca/ca (noncaracul)	42	27	64.3	
Fu/fu (fused)	50	27	54.0	*P* is between 0.02 and 0.03
fu/fu (nonfused)	52	39	75.0	

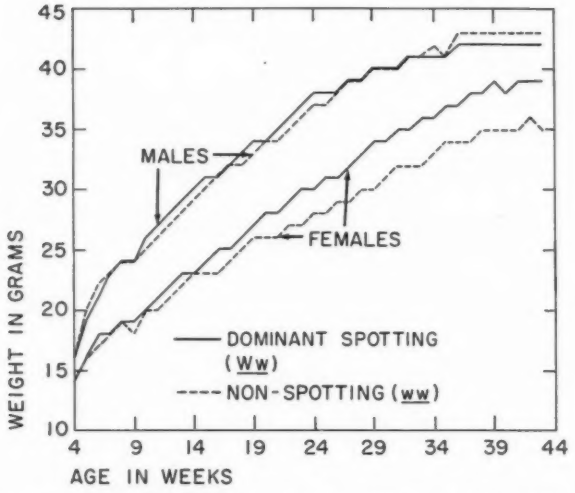
w/w. Of the *W/w* segregants 68 percent had tumors and of the *w/w* segregants 65 percent had tumors, thus failing to show a significant difference ($\chi^2=0.33$; P is between 0.5 and 0.7). There were 92 progeny of *Ca/ca* F_1 mice, 50 of which were *Ca/ca* and 42 were *ca/ca*. Sixty-two percent of the *Ca/ca* segregants and 64 percent of the *ca/ca* segregants had tumors, again failing to show a significant difference ($\chi^2=0.05$; P is between 0.8 and 0.9). There were 102 progeny of *Fu/fu* F_1 mice, of which 50 were *Fu/fu* and 52 were *fu/fu*. Fifty-four percent of the *Fu/fu* segregants had tumors, whereas 75 percent of the *fu/fu* segregants had tumors. Here the difference is significant ($\chi^2=4.9$; P is between 0.02 and 0.03). Thus, an association was shown between fused and susceptibility to pulmonary tumors induced with 1,2,5,6-dibenzanthracene. Whether this represents true linkage or the effect of *Fu* itself, however, cannot be determined from the present data.

This observation adds another gene on another chromosome, making a total of 5 chromosomes on which are located genes that influence susceptibility to pulmonary tumors. *W*, which in this test showed no relationship to susceptibility, is located on the same chromosome as hairless (*hr*), which has been associated with pulmonary tumors (*6*, *7*, *10*). In a former test, piebald, on the same chromosome with hairless but on the other side of hairless from *W*, was not shown to be linked with pulmonary tumors (*11*). These observations support the suggestion that the decrease in susceptibility associated with *hr* was due to the gene itself (*7*).

It is of interest that many of the genes associated with an effect on susceptibility to pulmonary tumors also have an effect upon body weight. Lethal yellow, which increases susceptibility to the tumors, increases body weight (*1*, *2*), whereas hairless, shaker-2, and flexed tail, which were associated with a decrease in susceptibility, decrease body weight (*4*, *6*, *7*). Vestigial tail, which was associated with a decrease in susceptibility to pulmonary tumors, however, had little effect on body weight (*8*), and certain other genes that have an effect on body weight have not been shown to have an effect upon susceptibility. Reduced weight produced by dietary restriction also has been associated with inhibition of pulmonary-tumor formation in mice (*12*, *13*).

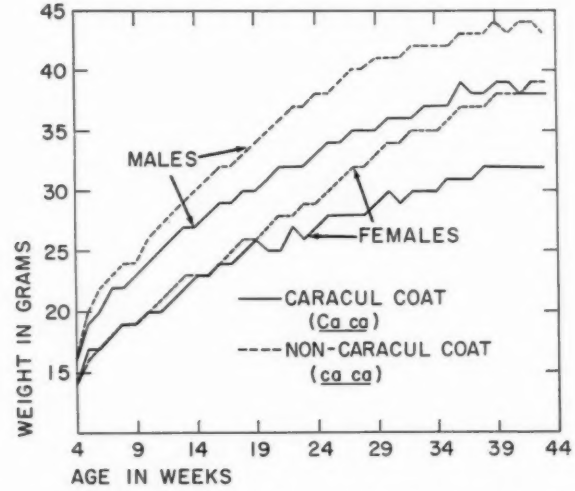
Growth curves for males and females of each group of segregants in the experiment reported herein are presented in text-figures 1, 2, and 3. The males with and without dominant spotting weighed approximately the same, but the females with dominant spotting weighed slightly more than those without spotting. Males and females with caracul coats weighed less than those with noncaracul coats, and males and females with fused tails weighed less than those with nonfused tails. Thus, the gene associated with a decrease in susceptibility to pulmonary tumors was associated with a like effect upon body weight, whereas the association of the other 2 genes with effects on body weight was not accompanied by a significant effect upon susceptibility to pulmonary tumors.

Other types of lesions were observed in a few of the animals in this experiment. Histologic sections of these lesions were made. Twelve

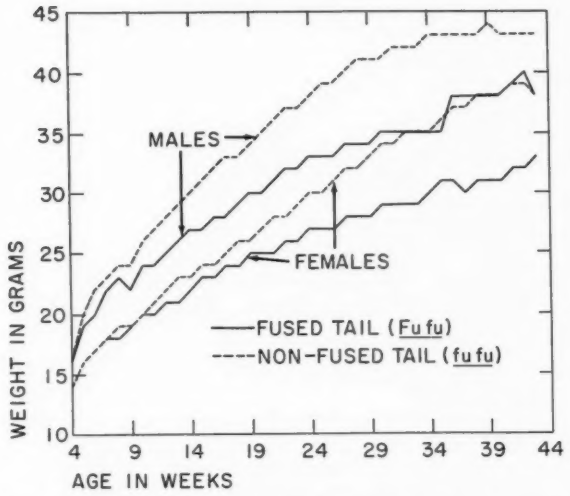


TEXT-FIGURE 1.—Weight curves for dominant spotting and nonspotting segregants.

animals (7 females and 5 males) developed sarcomas; 1 male, a lymphocytic neoplasm; 1 male, a plasma-cell neoplasm; and 4 females developed mammary gland tumors.



TEXT-FIGURE 2.—Weight curves for caracul and noncaracul segregants.



TEXT-FIGURE 3.—Weight curves for fused and nonfused segregants.

Summary

In the outcross resulting from crossing (A × WCaFu)F_1 and (WCaFu × A)F_1 animals with strain 1194, the segregants that were fused were less susceptible to induced pulmonary tumors than were the segregants that were nonfused. No significant difference in susceptibility was observed between the segregants with dominant spotting and those without spotting or between the caracul and noncaracul segregants.

Growth curves showed that the average weight of mice that were fused was less than that of mice that were nonfused and the mice that were caracul weighed less than those that were noncaracul. There was no difference between the average weights of the males with and without dominant spotting, but the females with dominant spotting weighed more than those without.

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An Approach to Chemically Defined Nutrients for Strain L Mouse Cells[1,2]

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The effectiveness of cultures of animal cells *in vitro*, for whatever purpose—the propagation of viruses (*1*), study of cellular processes such as mitosis (*2*), studies of morphogenesis (*3*), etc.—in every case depends upon control of the conditions of culture. The degree of precision required will vary with the nature of the particular objectives sought. Ideally all factors except the one under study should be controlled. Cellular nutrition is one of these factors.

Attempts have been made from time to time ever since 1912 [Lewis and Lewis (*4*)] to define the nutrients in which tissue cultures are grown. For many years the basic knowledge available and the techniques in general use were inadequate to permit attainment of this end. Since the major interest was at first largely morphological rather than physiological, and since complex organic nutrients consisting of combinations of serums and tissue extracts, capable of supporting rapid growth of a wide variety of cells, were available from a very early date (*5*), greater precision was for many years not essential for effective work, though still desirable. With the progressive shift in recent years toward physiological questions and the recognition that different cell types may have different nutritional requirements, not only for simple survival but also for typical expression of different functions, interest in defining the nutritional background has increased. It is hard to see how normal thyroxine secretion by thyroid cells can be studied effectively in a nutrient lacking iodine or in which the iodine levels are unknown or subject to uncontrolled variation. Fell and Mellanby (*6*) have recently shown how closely dependent is the development of keratinized or of ciliated mucosal epithelium on the available levels of vitamin A.

Improvement upon the embryo extract : serum : balanced salt solution nutrient can be approached by progressive analysis of the biological complexes, as Fischer has done (*7*), or by rejection of these complexes in favor of more or less arbitrary combinations of defined chemicals whose nutritional value has been demonstrated in other types of work, or by some

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combination of these two methods. The second, more radical approach, was first used in recent years by White (*8*, *9*). In 1950, Morgan, Morton and Parker likewise introduced a completely defined maintenance solution for animal cells (*10*). Parker's "mixture 199" is commercially available today and has gained rather wide usage. These solutions, although capable of supporting fairly prolonged survival (up to 100 days or more) of certain types of cells, still admittedly fall short of being able to support continued increase in cell number and are certainly capable of improvement.

This paper has two purposes. The first is to show that, beginning with an embryo extract : serum : balanced salt solution and substituting fully defined synthetic solutions for the BSS, it is possible to reduce greatly the concentration of these unknowns without sacrificing the severe requirements for rapid proliferation. For many purposes, both embryo extract and serum can be entirely dispensed with. The second purpose is to show that, within the defined portion, the major groups into which ingredients have been separated, for convenience, are all essential for normal growth, so that further simplification cannot come through elimination of any of these groups.

Materials and Methods

Earle's strain L mouse cells were chosen for this study because they represent a well-defined standard strain of single-cell origin, capable of cultivation directly on glass without supplementary substratum, and exhibiting a behavior which is already fairly well known. Cultures were grown in Carrel D-3.5 flasks. One ml. of fluid nutrient was employed. This was poured off and replaced three times each week, but the cultures were not "washed." Growth was recorded on a scale of 1 to 5. "One" represents an average of not more than 5 cells per field under the low power ($\times$100) of the microscope. It is our experience that such a culture has only about a 1 to 1 chance of survival even in the best of nutrients. A value of "2" represents an average of 10 to 20 cells per field and should generally survive and grow if the nutrient is at all satisfactory. A value of "3" represents a coverage of about two thirds of the surface of the glass in any given field. "Four" represents complete coverage of the glass with a single layer of cells, while "5" represents complete coverage plus sufficient crowding to force some cells to pile up into a second or third layer. At a density of "5" the crowded cells become rounded, and many of those not in contact with the glass tend to float free of the substratum. Such a culture has reached approximate equilibrium and is ready to be divided. While this method of measuring growth is less precise and quantitative than that obtained by treating the cultures with citric acid, centrifuging, staining, and making nuclear counts, as recommended by Sanford *et al.* (*11*), it is sufficiently objective and reliable that several observers will consistently give the same values to a given series of cultures. It has proved adequate for the purposes of this study.

Cultures having a density value of "5" were divided by scraping the

bottom of the flask with a curved spatula without removing the fluid, drawing up the resulting 1 ml. of cell suspension into a pipette and ejecting it forcibly six times to break up clumps of cells, and finally transferring the suspension in a series of equal parts, usually 3 to 6, to fresh flasks previously charged with nutrient. If the content of a flask with a value of "5" is so divided among 6 flasks, each subculture will have a total initial volume of 1.17 ml. (1+⅙) and will generally have an initial cell density of "2" or "3." Cultures so set up are quite uniform. In a nutrient consisting of 20 percent chick-embryo extract (1:1) : 40 percent horse serum : 40 percent Earle's balanced salt solution, such a culture will increase from a value of "2" to "5" in 5 to 7 days. More rapid growth than this is undesirable in a study of this sort, since the work required for maintenance becomes excessive and minor differences in nutrient value may be masked.

The experiments presented here consisted of replacing the usual balanced salt solution with one or another of the defined synthetic maintenance nutrients developed in this laboratory (*8*, *9*, *12*), reducing the concentration of one or both of the biological complexes (serum or embryo extract) to such a level that proliferation is just sufficient to maintain the density of the culture, and then modifying the synthetic portion in various ways likely to increase the proliferation rate over this base line. Throughout this paper the nutrients will be designated by their content of embryo extract, serum, and defined nutrient or salt solution in that order. Earle's standard nutrient is thus 20EE:40HS:40BSSE. Similarly, nutrient 2EE:2RS:96W_{II} would contain 2 percent chick-embryo extract (1:1), 2 percent rabbit serum in place of horse serum, and 96 percent of White's W_{II} solution.

The W_{II} solution employed here is a modification of an earlier nutrient described some years ago [White (*9*)]. It is made up from eight stock solutions as follows:

1. Carbohydrate

Fructose	17 gm.
Phenol red (0.01% sol.)	10 ml.
Water	to 100 ml.

Adjust to *p*H 7.4 with 0.1 *N* NaOH. Sterilize by filtration through a Selas porcelain candle, porosity 0.03.

2. Salts

NaCl	14 gm.
KCl	750 mg.
$MgSO_4$	550 mg.
$Ca(NO_3)_2 \cdot H_2O$	420 mg.
Water	to 100 ml.

Sterilize either in autoclave or by filtration.

3. Buffer

$NaHCO_3$	2.2 gm.
Na_2HPO_4	230 mg.
KH_2PO_4	104 mg.

Phenol red (0.01% sol.)	10 ml.
Water	to 100 ml.

Sterilize by filtration.

4. Iron

$Fe(NO_3)_3 \cdot 9H_2O$	2.75 mg.
Water	to 100 ml.

Sterilize in autoclave or by filtration.

5. Amino acids (excepting cysteine HCl and glutathione—see solution 7).

A. To 30 mg. cystine add 1 ml. H_2O, 1 ml. 1 percent phenol-red solution, and about 0.5 ml. 1 *N* HCl; warm, adding water, shaking, decanting, and adding more water until completely dissolved. Not more than 50 ml. should be required for complete solution.

B. Dissolve successively in a minimum of water (about 50 ml.) 560 mg. L-glutamic acid, 240 mg. L-aspartic acid, 624 mg. L-leucine, 416 mg. DL-isoleucine, 200 mg. L-phenylalanine, 520 mg. L-methionine, 520 mg. L-threonine, 520 mg. DL-valine, 624 mg. L-lysine HCl, 312 mg. L-arginine HCl, 104 mg. L-histidine HCl, 200 mg. L-proline, and 400 mg. glycine, adding 1 ml. of 1 percent phenol red and sufficient 1 *N* NaOH to bring to *p*H 7.6.

C. Dissolve 160 mg. L-tryptophan in 50 ml. water, *without heat.* To this, add A and B, make up to 200 ml. with water, adjust to *p*H 7.6, and sterilize by filtration.

6. B vitamins (except B_{12}—see solution 8).

A. Dissolve successively in 30 ml. of water 10 mg. thiamin, 10 mg. riboflavin, 10 mg. Ca-pantothenate, 50 mg. pyridoxine, 50 mg. nicotinic acid, 50 mg. *i*-inositol, 50 mg. *β*-alanine, 100 mg. choline HCl, and 10 mg. *d*-biotin.

B. Dissolve in 30 ml. of water 10 mg. folic acid (pteroylglutamic acid) and 10 mg. $NaHCO_3$. Mix A and B and make up to 100 ml. with water. To 40 ml. of this add 160 ml. of water. Sterilize by filtration.

7. A and C vitamins (plus cysteine HCl and glutathione).

A. Dissolve in 100 ml. ethanol 10 mg. vitamin A (free alcohol) and 10 mg. *β*-carotene.

B. Dissolve 10 mg. ascorbic acid, 20 mg. glutathione, and 20 mg. cysteine HCl in 100 ml. water.

C. Dissolve 10 mg. phenol red in 100 ml. of water. Mix 2 ml. of A, 10 ml. of B, and 2 ml. of C; make up to 100 ml., adjust to *p*H 7.4, and sterilize by filtration.

8. Vitamin B_{12}.

Dissolve 5 mg. vitamin B_{12} in 100 ml. water. Dilute this 1:100. Store in refrigerator without sterilization.

The control solution (W_{II}) consists of 5 ml. each of solutions 1 to 8 plus 60 ml. of water. It contains the following final concentrations in milligrams per liter:

Fructose	8, 500	Glycine	100
NaCl	7, 000	Cysteine HCl	1
KCl	375	Glutathione	1
$MgSO_4$	275	Thiamin	1
$Ca(NO_3)_2 \cdot H_2O$	210	Riboflavin	1
$NaHCO_3$	1, 102. 5	Ca-pantothenate	1
Na_2HPO_4	115	Pyridoxine	5
KH_2PO_4	52	Nicotinic acid	5
$Fe(NO_3)_2 \cdot 9H_2O$	1. 375	*i*-Inositol	5
L-cystine	7. 5	β-Alanine	5
L-glutamic acid	140	Choline HCl	10
L-aspartic acid	60	*d*-Biotin	1
L-leucine	156	Folic acid	1
DL-isoleucine	104	Ascorbic acid	0. 5
L-phenylalanine	50	Vitamin A	0. 1
L-methionine	130	β-Carotene	0. 1
L-threonine	130	Vitamin B_{12}	0. 025
DL-valine	130	Phenol red	4
L-lysine HCl	156	Ethanol	1
L-arginine HCl	78		
L-histidine HCl	26		
L-proline	50		
L-tryptophan	40		

The original culture received from Earle[3] in April 1953, consisted of a single T-60 flask with a perforated cellophane insert, bearing the code number "929-126-114." Upon receipt, the flask was placed in the incubator at 37.5±1° C. and left for 24 hours to permit reattachment of the cells that had broken loose in shipment. Half the nutrient was then removed and replaced with a 20EE:40HS:40BSSE nutrient (Earle's standard nutrient). Two days later the flask was scraped thoroughly and 5 ml. of the cell suspension withdrawn and inoculated, without washing, in 0.5 ml. aliquots into 10 Carrel D-3.5 flasks, each containing 1.0 ml. of 20EE:40HS:40BSSE nutrient. Thereafter, nutrient was renewed in full three times weekly (Monday, Wednesday, Friday), but without washing. When these cultures had reached a density of "5" (in about a week), they were again divided, providing a source of cells both for stock cultures and for nutrient experiments.

Experimental

White (*8*, *9*) and Waymouth (*12*) have developed several synthetic nutrients in this laboratory. These nutrients contain amino acids, vitamins, and other defined metabolites and can be used to make, for

[3] We wish to thank Dr. Earle for his kindness in furnishing this initial culture.

example, a 20EE:40HS:40W_{II} nutrient. When Earle's balanced salt: dextrose solution was replaced in this way and the nutrient was supplied to strain L cells that had been freshly transferred from Earle's standard nutrient, it soon became apparent that this combination of defined nutrient with the standard organic complexes provided too rapid growth for our purposes. Growth reached a "5" level in only 2 to 4 days. Progressive reduction finally resulted in complete elimination of the embryo extract and reduction of the serum to 5 percent. Such a 0EE:5HS:95W_{II} nutrient will support growth of strain L cells at a rate nearly if not quite equal to that obtained in the original 20EE:40HS:40BSSE nutrient (fig. 1). This 0:5:95 nutrient, in which the W_{II} formula is used as diluent, has now been used in this laboratory for more than a year for the routine maintenance of stock cultures of strain L cells. In practice, we have routinely substituted rabbit serum for horse serum since the former is available from our own animals. Horse serum is, however, equally effective. Fructose has also been substituted for dextrose (unpublished experiments), and the salt solution has been modified by doubling the buffer concentration (both phosphate and bicarbonate) over the 1949 formula (*13*).

The synthetic solution W_{II} is thus capable of replacing 11/12ths of the nutritional effects commonly attributed to the serum and embryo extract. Apparently the embryo extract is completely replaced, for not only can embryo extract be dispensed with but partial replacement of the serum with embryo extract to give a 2EE:2RS:96W_{II} solution instead of a 0EE:5RS:95W_{II} gave distinctly inferior results. Reduction of the serum to 0EE:2RS:98W_{II} gave cultures which maintained themselves indefinitely with mitoses just slightly more than balancing cell losses, so that it required 8 weeks to reach a "5" level (fig. 2). The cells were excellent morphologically, without marked change from their appearance in Earle's nutrient. Upon transfer to a 0EE:5RS:95W_{II} nutrient, such cultures immediately resumed rapid growth. This is the sort of culture that we consider ideal as a base line against which to test nutrient variants, since it is highly sensitive to relatively minor changes in nutrient effectiveness.

In a 0EE:1RS:99W_{II} nutrient there was no over-all increase in cell number, and cultures were quite uneven (fig. 3). Nevertheless, mitoses were by no means rare after 8 weeks in such a nutrient; there was no regular falling off in general density, and the growth pattern, although uneven, was clearly superior to that when serum was entirely omitted (fig. 4).

This progressive reduction has been carried still further by eliminating not only the embryo extract but the serum as well to give the general formula 0:0:100 (fig. 4). If strain L cells are established in a 0EE:5RS:95W_{II} nutrient and allowed to grow for 72 hours, they will attain a uniform density of about "4." If the nutrient is then poured off and replaced by a 0:0:100W_{II} solution, mitoses continue to be visible in considerable number for 4 or 5 days, through one subsequent renewal of nutrient. Thereafter, mitoses become rare and the cells in contact with the glass take

on a characteristic flat, excessively stellate form with many long, slender processes. Cells not in contact with the glass tend to round up, and some float free. Some cells, however, continue to migrate rapidly, undergoing constant "boiling," with rapid formation of large, transparent vesicles. These are clearly seen after 3 or more weeks. The cell density is maintained with little reduction and little general disintegration for about 2 weeks. Thereafter there is a progressive deterioration. Cells tend to clump, some become glassy in appearance, others become highly vacuolated, and still others shrivel and die. Cell number decreases. Nevertheless, the cells which survive, and there are many of them, retain their full viability. When transferred to a 0EE:5RS:95W_{II} nutrient after 2, 3, 4, and even 6 weeks in a 0:0:100W_{II} nutrient, they resume active growth promptly (figs. 5 and 6). All of our current strain L cultures have been subjected repeatedly to more or less prolonged periods on fully defined nutrients. All have had at least 200 days without serum or embryo extract (60 to 70 renewals of nutrient) including 2 periods of 4 weeks each, although not 200 consecutive days of "starvation."

To determine whether further simplification could be obtained by eliminating any of the major groups of nutrient constituents other than serum advantage was taken of the eight stock groups used in making up the W_{II} solution. A series of nutrients was prepared based on the 0:2:98 nutrient found to represent the minimum serum level capable of sustaining a continued increase in cell number. The 2 percent rabbit serum, salts (stock solution 2), and buffer (stock solution 3) were retained in all nutrients. The other six stocks—1) sugar (fructose), 4) iron (ferric nitrate), 5) fifteen amino acids but without cysteine HCl and glutathione, 6) and 8) containing all the B vitamins used, and 7) A and C vitamins plus cysteine HCl and glutathione, were omitted in turn. The deficient nutrients (minus sugar, minus iron, minus amino acids, minus B vitamins, minus A and C vitamins) all contained serum and the required salts and all but one of the above constituent groups. It is recognized that traces of "omitted" constituents may have been present as impurities in those supplied. In most cases such contamination is probably insignificant, although contamination by trace elements such as copper might be important. Six cultures were established in each nutrient, and nutrients were renewed three times a week until all cultures of a series were dead or until they had been maintained for 8 weeks (24 renewals).

In the deficient nutrients, those lacking sugar were the first to break down (fig. 8). Within 3 days the cells became shriveled and granular, and many were dead. No mitoses were visible after 5 days. A few cells were still alive at 10 days, but all were dead by 14 days (6 renewals of nutrient). A striking feature of those cells still living after 12 days (fig. 8) was the exaggerated development of the undulating membrane, which was extremely active, as if the cells were desperately taking in quantities of water in an effort to obtain sufficient amounts of the missing sugar. They behaved in this respect like "diabetic" cells.

The next cells to succumb were those lacking the B vitamins (fig. 9).

Cells in this nutrient quickly became large and excessively stellate with many long, slender processes. A few mitoses were visible up to 17 days; all cells were dead at 27 days. Figure 9 shows such a culture at 12 days. Some cells were still flat and highly stellate, but more had already rounded up, and the granular debris from disintegrated cells was visible in some places.

The third group of cells to succumb was the one lacking the amino acids (fig. 10). The effect of this deficiency was evident within 5 days, the cells becoming narrow and somewhat distorted, with few (usually 2 or 3—seldom more) processes. Nevertheless, some mitoses were evident up to 19 days, and occasional cells survived to 33 days.

The response in the absence of the A and C vitamins was interesting (figs. 11 and 13). The cells at first appeared to be larger and more active than in the control medium, and many normal-appearing mitoses were visible up to 33 days. Nevertheless, there was a gradual and increasing tendency for cells to clump together and for the cytoplasm of those which did not so clump to become highly vacuolated (fig. 13). Although many cells survived the entire 8 weeks of the experiment, the over-all cell density had dropped to less than "1" as compared to "3" in the initial cultures and "5" in the final controls (fig. 2).

Those lacking iron were likewise interesting (figs. 7 and 12). Like those lacking A and C vitamins, the cells were large and appeared in excellent condition. Many survived the entire period. But unlike the A- and C-deficient cells the mitotic frequency quickly dropped to a low level and remained low so that, with gradual loss of cells, the cell density decreased although cell quality was maintained. By the end of 8 weeks, the density level was a scant "3" (fig. 12), although mitoses were still visible and the cells, while perhaps somewhat more stellate than usual, were otherwise in excellent condition.

Discussion

It is clear from these results that if a defined nutrient mixture, W_{II}, is employed in place of the usual balanced salt solution, strain L cells can be maintained and fairly high proliferative levels sustained in nutrients whose undefined complex components are markedly reduced as compared with those commonly used. The embryo extract can be dispensed with entirely and the serum can be reduced to not more than 2 percent. This represents a 30:1 reduction of undefined ingredients (serum, embryo extract), with a corresponding increase of the defined portion from 40 to 98 percent.

Within this defined portion *none* of the major constituent groups of substances can be dispensed with. Sugar, amino acids, B vitamins, A and C vitamins, and iron are all necessary for satisfactory cell proliferation in a 2 percent serum nutrient. Two percent of serum does not contain adequate supplies of any of these groups. We cannot simplify our nutrient by the omission of any of these groups *in toto*. Simplifications can be made only by breaking down the amino-acid group, the B vitamin

group or the A and C vitamin groups into subgroups or to their single constituents. The total level of amino acids and the levels of sugar, salts, and iron were established previously as "optimal" for chick cells (*8*, *9*). It seems doubtful that these will change drastically upon further study, although we have found it desirable to double the concentration of buffer (*13*). No such "optimal" levels were established, however, for the vitamins or for the individual amino acids, the levels used being chosen quite arbitrarily from information in the general literature of microbiology. Our W_{II} nutrient contains vitamin concentrations from 2 to 100 times those given in Parker's 199 (*10*) and 703 (*14*) formulas. These will have to be re-examined in detail.

It is possible that additions, deletions, or readjustments can be made within these groups with benefit to the over-all result. It seems more likely, however, that at this point attempts to analyze the residual 2 percent of serum might prove fruitful.

Although it is not yet possible to maintain stock cultures of strain L cells at a rate of rapid increase without the use of any unknown, it is possible to carry on some experiments—fairly prolonged ones—in completely defined nutrients. Cells can be maintained for at least 6 weeks in such defined nutrients without loss of viability, as is evident from their prompt resumption of growth upon transfer to more "complete" nutrients. In experiments which do not require more than 10 days or 2 weeks for completion, we thus have at hand the possibility of maintaining the cells under conditions which are, nutritionally, under complete control. Virus cultivation represents an application in which a period of 2 weeks without renewal of cell colony is seldom required, although not all viruses will multiply in the absence of proliferative increase. Metabolic studies likewise seldom employ long experimental periods, although there are, of course, exceptions. Thus both are types of experiments for which defined nutrients might be valuable.

The nutrient formula W_{II} and, to an even greater extent, Waymouth's formula "C" (*12*), which is also suitable for use in a 0:5:95 nutrient for strain L cells are in both cases much simpler and less expensive than Parker's formulas 199 and 703 (*10*, *14*), which are currently widely used. We are in the process of improving these nutrients by addition, substitution, and deletion and have every expectation of eventually being able to eliminate entirely the need for the use of biological complexes and of being able, thus, to put the cultivation of animal cells on a sound, defined nutritional basis.

It is perhaps noteworthy that formula W_{II}, which has proved suitable for strain L mouse cells, was originally developed as a maintenance solution for chick tissues. The earlier formula (*8*) has also been used as a diluent in preparing solutions for perfusion of mammalian organs (*15*), for studies of human uterine decidua (*16*), and for studies of the effect of tumors and tumor extracts on development of the nervous system of chick embryos (*17*). Waymouth likewise found (*12*) that slight changes in salt and amino-acid ratios sufficed to adapt a synthetic nutrient for

use with chick heart, chick lung, or mouse fibroblasts. While it seems probable that different cells and organs will require different nutrients for the optimum expression of their various functions, which are scarcely approached by the formulas now available, it also appears that we now do have one or more formulas around which we can begin to build such optimal nutrients.

There is one paper in the literature which appears to represent results somewhat at variance with those presented here. Evans *et al.* (*18*), using standardized replicate cultures of strain L cells in a series of nutrients which included Parker's mixture 199 and White's 1949 nutrient (*9*), concluded that in these two defined nutrients the number of viable cells decreased rapidly, so that the cultures no longer contained viable cells after 11 days. Their procedures involved differences from those used here, two of which we believe to be important. First, their strain L cells were washed thoroughly in saline before inoculation, while ours were not only transferred directly from 0:5:95 nutrient without washing, but were allowed 3 to 5 days in 0:5:95 nutrient after transfer, during which to become established, before presenting them with the defined nutrients. These authors thus failed to take advantage of the gradual adaptation of cells and medium, the importance of which, in critical experiments, they themselves had pointed out earlier (*19*). And second, if one may judge from their photographs and figures (text-fig. 1 and plate 85, fig. 4) their initial inoculum was at a density approximately equivalent to our density "3," while our cultures, when presented with a $0{:}0{:}100W_{II}$ nutrient, had a density of "4" to "5." As the same authors have pointed out elsewhere (*20*), survival in any nutrient is in part dependent on size (density) of inoculum. We have found this factor to become increasingly important as the degree of proliferative effectiveness of the nutrient is decreased. In both of these respects the authors cited have imposed conditions which are not fully consonant with their own previous recommendations and which are, we believe, unnecessarily severe. These differences may well explain why our more favored cultures have given better results.

The fact that strain L cells, once established in flasks, can survive in completely defined nutrients of relatively simple formula for 6 or more weeks (18 or more complete renewals of nutrient) without loss of viability, is a step forward from which we can safely expect further advances.

Summary

Strain L mouse cells are now cultivated routinely in this laboratory in nutrients consisting of any one of several fully defined synthetic solutions supplemented with 5 percent rabbit serum, without embryo extract. In these nutrients they maintain a proliferation rate equal to that in Earle's standard 20EE:40HS:40BSSE nutrient. They continue to proliferate slowly with only 1 percent serum. In a completely defined nutrient, without either serum or embryo extract, proliferation ceases but cells survive for at least 6 weeks and resume growth within a few days upon

transfer to a supplemented nutrient. Within the defined synthetic solution all of the eight groups of constituents are essential for normal proliferation.

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All figures represent living cultures of strain L mouse cells grown in Carrel flasks in nutrients consisting of various modifications of fully defined synthetic nutrient (W_{II}) supplemented with designated levels of rabbit serum but without embryo extract. Photographs were made with a Zeiss × 12 Photo-ocular and Bausch and Lomb × 10 objective, Eastman XX Panatomic film, and a Wratten 58 filter. All figures are reproduced at magnification of × 100.

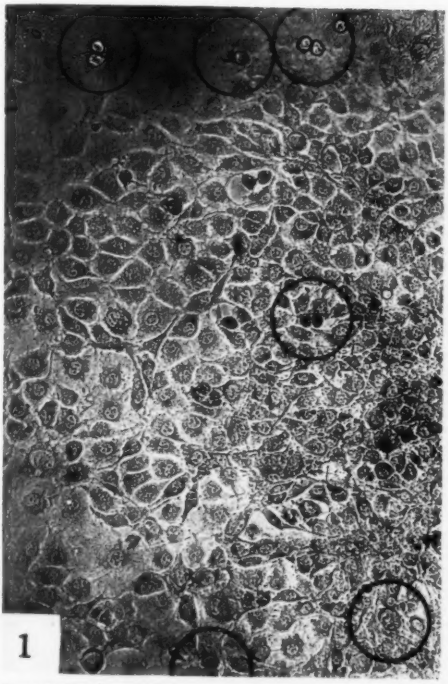
PLATE 65

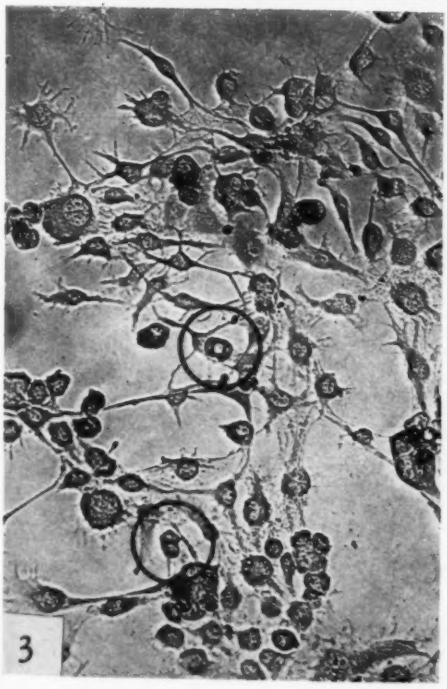
FIGURE 1.—Five percent serum, 9 days.

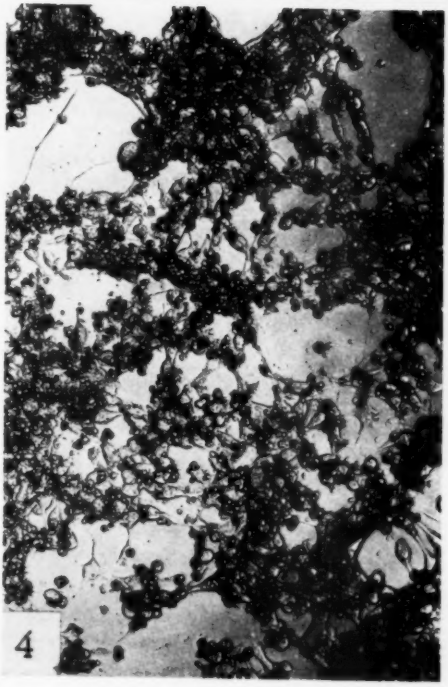
FIGURE 2.—Two percent serum, 8 weeks.

FIGURE 3.—One percent serum, 8 weeks.

FIGURE 4.—No serum, nutrient completely defined, 6 weeks.







White
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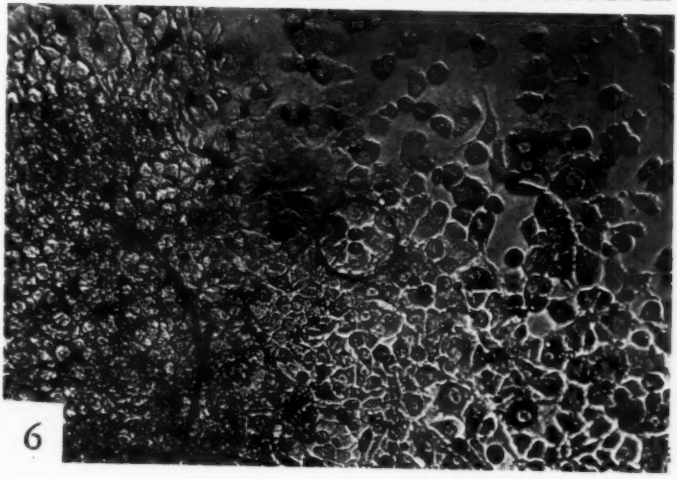
PLATE 66

FIGURE 5.—Four weeks in completely defined nutrient followed by 5 days in 5 percent serum (*cf.* figs. 1 and 4).

FIGURE 6.—Six weeks in completely defined nutrient followed by 4 days in 5 percent serum (*cf.* figs. 1 and 4).

5



6

White

PLATE 67

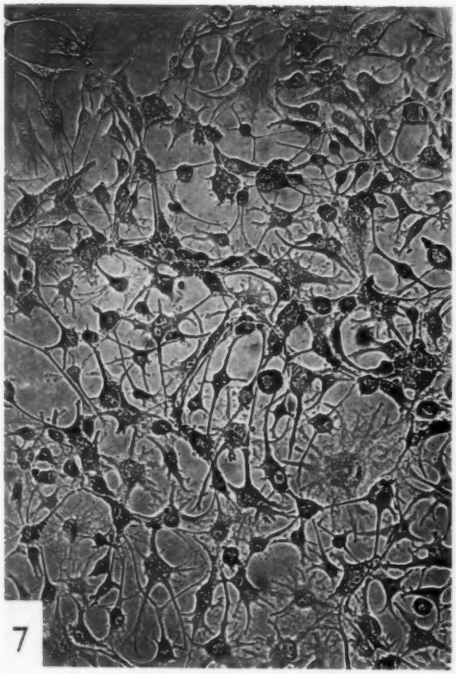
Figures 7 to 13 represent cultures in a 2 percent serum nutrient modified by omission of designated constituents or groups of constituents.

FIGURE 7.—Iron omitted, 12 days.

FIGURE 8.—Sugar omitted, 12 days. Note the greatly developed undulating membrane.

FIGURE 9.—B vitamins omitted, 12 days.

FIGURE 10.—Amino acids excepting cysteine HCl and glutathione omitted, 12 days.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8

9

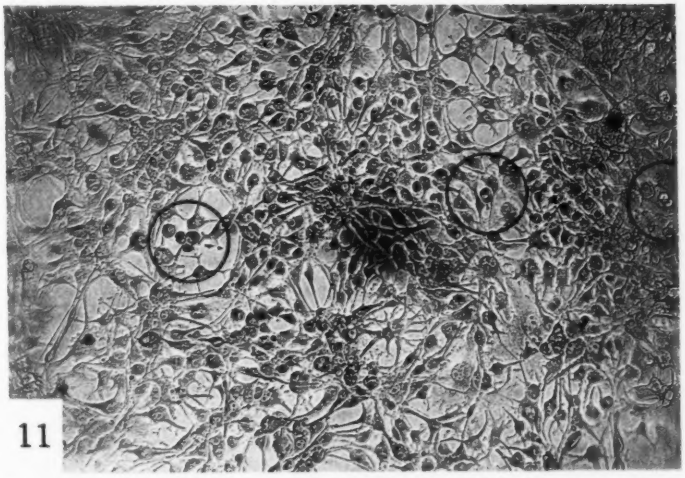
10

PLATE 68

FIGURE 11.—Vitamins A and C, β-carotene, cysteine HCl, and glutathione omitted, 12 days.

FIGURE 12.—Iron omitted, 8 weeks.

FIGURE 13.—Vitamins A and C, etc. omitted, 8 weeks.



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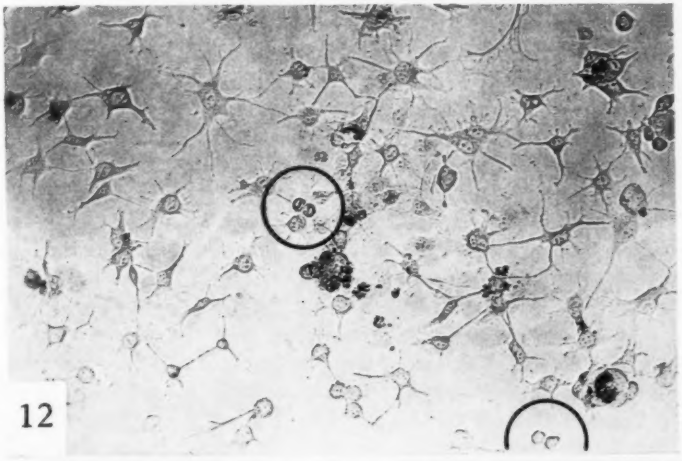

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The Effect of Serum Fractions on the Proliferation of Strain L Mouse Cells *in Vitro*[1]

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Introduction

The purpose of this study was to examine those components of the large molecular portion of serum that appear to be essential for the rapid proliferation of strain L mouse cells *in vitro*, with the ultimate objective of isolating a component that could be added to a chemically defined medium for the purpose of increasing the rate of cell proliferation. Although several chemically defined, protein-free media have been described in which proliferation of strain L (clone 929) cells occurs (*1*, *2*), in none of these is the extent of cell proliferation comparable to that of cells grown in supplemented washed horse-serum residue (*3*).[3] Washed serum residue alone fails to support cell survival and proliferation, but when supplemented with either amino acids and vitamins or an ultrafiltrate of chick-embryo extract (*3*), good proliferation occurs. When supplemented with both, the rate of proliferation of strain L cells appears to be optimal. An attempt has been made to determine whether any fraction of washed serum residue can be substituted in this system for the whole serum residue without decreasing the optimal rate of cell proliferation.

Before undertaking the task of fractionating the horse-serum proteins, commercially prepared bovine plasma and serum fractions were tested on the cells in the hope of finding a cheap and reproducible protein fraction that could be obtained commercially. Since none of these fractions proved satisfactory, apparently because of partial denaturation of the proteins and use of toxic precipitating or purifying agents, horse serum was fractionated by the Cohn low-temperature procedure, Method 10(*4*). This procedure was chosen as the one most likely to yield good-quality protein, undenatured and nontoxic to the cells.

Experimental Procedures

Assay procedure.—All serum fractions were tested on replicate cultures of mouse cells, clone 929 of strain L (*5*, *6*). Replicate cultures of washed

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[3] Washed serum residue refers to the large molecular portion of horse serum cleared of its freely diffusible, small molecular-weight substances by ultrafiltration.

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cells were prepared according to methods previously described (*7*). The test media were quantitatively renewed thrice weekly, and the cultures were gassed with 5 percent CO_2 in air to adjust the *p*H to 7.3 at each renewal. At weekly intervals 4 or 5 cultures from each experimental group were selected at random for microscopic observation and the enumeration of cell nuclei. The number of cell nuclei in each culture was determined by the procedure reported (*8*), with the following modifications: 1) The adjustable pipette clamp on the Bryan-Garrey rotor was reset to angle the pipettes more sharply and thus increase the amplitude of motion of the modified glass bead used to agitate the nuclear suspension in the pipette bulb. The angle between the axis of the pipette and the axis of the rotor shaft was increased from 12 to 22°. 2) Pipettes in which the modified glass stirring bead had been broken were discarded, since broken beads were found to mutilate the cell nuclei. 3) In the last three experiments, each suspension of nuclei was counted by one operator instead of three; however, each inoculum sample was counted by three operators. The standard error of the mean and percent error for the inoculum samples and for the cultures in each experimental group were calculated by the short method of Mantel (*9*).[4]

Assay medium.—The control lots of serum and the serum fractions were tested in a basal medium consisting of solution NCTC 102 (*10*), ultrafiltrate of chick-embryo extract or of whole egg prepared according to procedures described (*11*, *12*), and Earle's balanced saline (table 1). The solution NCTC 102 contained the materials of Morgan, Morton, and Parker's mixture 199 (*13*) except that the amino acids were replaced by an amino acid mixture based on analyses of ultrafiltrates of horse serum and embryo extract (*14*, *15*). The amino acids were used at ten times the concentration, as determined in a solution of 40 percent horse serum, 20 percent embryo extract (1:1), and 40 percent Earle's saline. The final concentration in the medium in which the cells were growing was approximately 2.4 times the concentration in whole horse serum. In

TABLE 1.—*Composition of assay medium and protein concentration of serum or serum fractions tested*

Expt. No.	Composition of basal medium (% *by volume*)				Protein concentration of serum and serum fractions (*gm. per 100 ml. final medium*)*
	NCTC 102	Ultrafiltrate		Saline	
		Embryo extract	Whole egg		
1	40	20	—	20	1. 3
2	40	20	—	20	1. 3†
3	40	20	—	20	1. 3
4	40	20	—	20	1. 3
5	40	—	20	20	2. 6

*The protein solutions constituted 20 percent by volume of the final medium.
†For two exceptions see text.

[4] The authors wish to thank Mr. Marvin Schneiderman of the Biometry Section of this Institute for his advice regarding the statistical aspects of this study.

experiment 5, the ultrafiltrate portion was prepared from whole egg; this ultrafiltrate has been shown to have a growth-promoting effect for strain L cells equivalent to that of embryo-extract ultrafiltrate when used in this system (*12*).

In all experiments, except when indicated, the test fractions were added to the basal medium at the same protein concentration as the control lots of horse serum or washed serum residue. Whole serum rather than serum residue was used as a control in some of these experiments since it is known that the proliferation of strain L cells in whole serum in this system (*3*) approximates that of cells grown in horse-serum residue.

Preparation of serum.—The serum was obtained from healthy, middle-aged geldings by direct bleeding into a glass container in which the blood was stirred continuously with a glass rod. The stirring was continued for 3 minutes after bleeding ceased and the fibrin clot was lifted from the vessel.[5] The container was surrounded by ice and allowed to stand at 0° C. for 16 hours to let the cells settle. The serum was siphoned off and gassed with CO_2 to adjust the *p*H. Some serum lots were then centrifuged at 2,000 × g for 30 minutes, passed through a Selas #03 filter, and used as fresh serum. Other lots were frozen and stored at −10° C. for variable periods before use. When the frozen serum was to be used, it was thawed by immersing the container in water at 37° C. The thawed serum was centrifuged at 2,000 × g for 30 minutes to remove some sediment and precipitated lipids. This freezing and thawing process, which is routinely used in the preparation of serum for stock-culture medium, evidently separates some of the more labile lipid from lipoprotein combination. The lipid centrifuged out contained about 25 percent of the total cholesterol in freshly drawn ACD plasma from the same horses. A small amount of protein precipitate also was removed. Whether this protein moiety was some portion precipitated incident to the CO_2 gassing for *p*H adjustment, or a residuum from the fibrinogen removal with the clot, or whether it arose incidental to the freezing is not known. It is removed in the routine preparation of serum and, hence, must be a material which is not essential for good cell growth; it was not considered further.

Fractionation of serum.—The separation of protein components by Method 10 (*4*) was carried out in a room maintained at −10° C. Ordinarily a liter of serum was used for the first step of the fractionation so as to have adequate amounts of protein in the fractions to be assayed; an aliquot of each lot of serum was retained as an unfractionated control. The alcohol-acetate buffer needed for the combined precipitates I, II, and III of Method 10 was cooled to −5° C. overnight. The liter of serum was

[5] The demonstrated tendency of the clot not only to entrap proteins but to retain preferentially certain proteins more than others (*16*) has deterred workers from applying Method 10 to serum rather than to plasma (*17*). Because serum has been demonstrated to be a necessary and useful constituent of the medium for optimum growth of certain cell strains in this laboratory, it rather than plasma was taken as the starting material for fractionation. The use of serum meant that certain fractions of Method 10 would be lacking; *e.g.*, there would be no fibrinogen, little thrombin or plasmin, and by actual test the cholesterol was found to be 5 percent less than in ACD plasma. This latter finding possibly indicated removal of some of the lipoproteins by the fibrin clot, since Morrison (*16*) found that a portion of the β- and α-lipoproteins may be occluded by the clot.

then cooled to −1° C. and, just as the first ice crystals started to form, 4 liters of the acetate buffer were added slowly from a fine-tipped burette while the serum was stirred mechanically. An hour was allowed for the addition of the 4 liters after which period stirring was continued for an additional 20 minutes; the globulin precipitate was collected by centrifugation at 2,000 × g at −5° C. Aliquots of the supernatant fluid were taken for the following estimations: protein by the biuret method (*18*), cholesterol (*19*), and *p*H (*A*).

Fractions IV, V, and VI of Method 10 were now in the supernatant. The difficulties of removing zinc ions from protein to be used on living cells precluded the use of Method 10 for further fractionation of the supernatant. Accordingly, the supernatant fluid was transferred to a collodion membrane and ultrafiltered in a modified Simm's ultrafiltration apparatus (*11*) to remove the alcohol and acetate, which were gradually replaced by Earle's balanced saline. The ultrafiltration introduced the dangerous step of gradually warming the protein solution from −5 to +5° C. in the presence of 19 percent ethanol. One lot of albumin (lot 2 of experiment 5) was removed from the supernatant by the procedure of the Cohn Method 6 (*20*) which involved precipitation with 40 percent alcohol. The sedimented protein was then taken up in Earle's saline at −1° C. and washed by ultrafiltration to remove alcohol and acetate. All ultrafiltrations were carried out at 5° C. under pressure of 5 percent CO_2 in N_2. The solution of fractions IV, V, and VI was ultrafiltered until the washings were free of alcohol and gave a negative test for α-amino acids with ninhydrin (*21*); the period necessary for the ultrafiltration was generally about 4 days. Fractions IV, V, and VI, containing mainly the albumins and the α-globulins, were not separated further (except for lot 2, experiment 5 mentioned above) and in this paper will be referred to as fraction IV.

When the gross-globulin precipitate was spun down by centrifugation as noted above, the moist paste containing fractions I, II, and III was found to contain approximately half of the total serum proteins—*i.e.*, 1 liter of the buffer-serum mixture (1 part of serum to 4 parts of buffer) yielded about 60 grams of moist paste containing 7 grams of protein. This precipitate was extracted a second time to remove contaminating albumins. During the second extraction about 6 percent of the protein remained in the wash solution and probably represented chiefly contaminating albumins and α-globulins. This portion was not added to fraction IV but was discarded because the resulting volume increase in the albumin fraction would further lengthen the time necessary for ultrafiltration[6] and concentration of this fraction. It is assumed that the discarded portion had a composition similar to the supernatant from the first separation, though more dilute. Even after the second extraction of the gross-globulin fraction to remove contaminating albumins, there remained a small amount of material behaving like albumin when

[6] It has been established that in adequate media (*22*) certain pseudomonads, coliforms, and achromobacters can thrive even at 5° C. The ultrafiltration technique is nonsterile; therefore length of time of filtration was held to a minimum.

subjected to the Green and Wade precipitation for albumin (*23*) and the Durrum paper-electrophoresis procedure (*24*). Further tests indicated that on fractionation this contaminating albumin was carried into the γ-globulin fraction.

When the gross globulin was to be used with no further fractionation, the precipitate at −5° C. was dissolved in Earle's saline chilled to the point of incipient ice formation; the alcohol and acetate were removed by ultrafiltration, and saline washes were continued for 2 to 5 days until the ultrafiltrate gave a negative test for α-amino acids with ninhydrin. The gross globulin (containing fractions I, II, and III) is henceforth referred to as fraction I.

The γ-globulins (fraction II) were extracted from the combined precipitates of fractions I, II, and III with the glycine buffer of Method 10. The large quantity of glycine in solution was removed by ultrafiltration; the quantity removed was measured by the colorimetric ninhydrin procedure for glycine in the ultrafiltrate (*21*). The initial step of ultrafiltration involved what also is regarded as a dangerous exposure of the γ-globulin to 15 percent alcohol at 5° C. for several hours during the ultrafiltration. It was usually necessary to continue washings and ultrafiltration for 9 or 10 days before the ultrafiltrate gave a negative test with ninhydrin and the fraction could be considered cleared of all freely diffusible materials other than those added by the saline.

The fraction containing precipitates I and III of Method 10, after γ-globulin extraction, was taken up in Earle's saline at −1° C. and ultrafiltered with saline washings for about 5 to 8 days until the ultrafiltrate was free of alcohol and glycine. This residual globulin fraction, containing mainly the β-lipoproteins, is henceforth referred to as fraction III.

Washed serum residue was prepared by ultrafiltration of horse serum with the addition of Earle's saline. Washings were usually continued for 1 to 2 days until the ultrafiltrate gave a negative test for α-amino acids with ninhydrin. The negative ninhydrin test was used as perhaps the simplest test for removal of ordinary diffusibles.

After the serum residue and all serum fractions were washed, they were concentrated to a suitable protein content by further ultrafiltration; the preparations were then centrifuged at 2,000 × g for 30 minutes and passed through a Selas #10 and a Selas #03 filter under positive pressure of 5 percent CO_2 in N_2. Samples of all filtered solutions were analyzed for protein by the biuret method (*18*). The solutions were used fresh and were stored in sterile state at 5° C. in sealed tubes during the course of the experiment.

The protein content of horse serum and horse-serum fractions before ultrafiltration and washing of fractions is presented in table 2. The composition of the fractions of experiment 5 as measured by paper-electrophoretic examination is presented in table 3. It was not possible to demonstrate electrophoretically any difference between these fractions from serum and the corresponding fractions from plasma.

TABLE 2.—*Protein content of horse serum and horse-serum fractions before ultrafiltration and washing of fractions*

Horse serum or serum fraction	Serum protein concentration (*gm./100 ml.*)
Horse serum	7. 5*
I (gross-globulin)	3. 1
II (γ-globulin)	1. 9
III (globulin-residue)	1. 2
IV (gross-albumin)	4. 8

*Comparison with ACD plasma indicated that 320 mg. per ml. was removed with the fibrin clot.

TABLE 3.—*Composition of horse-serum fractions of experiment 5 as measured by paper-electrophoretic examination*

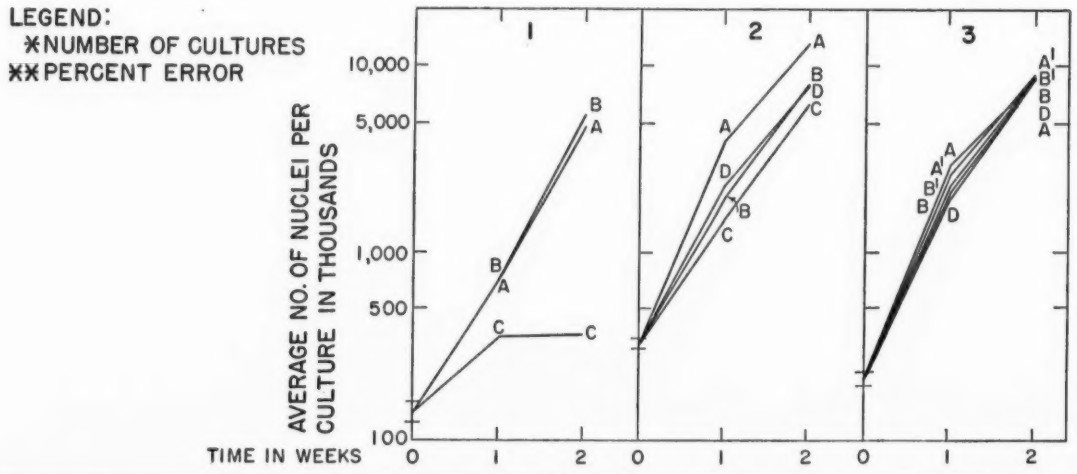
Protein components	Composition of serum fractions (*gm. per 100 ml.*)			
	I (Gross globulin)	II (γ-globulin)	III (Globulin residue)	IV (Gross albumin)
Albumin	*	*	—	2. 60
α-1-globulin	—	*	—	0. 20
α-2-globulin	—	*	—	0. 10
α-3-globulin	0. 50	*	0. 50	—
β-1-globulin	—	—	—	0. 80
β-2-globulin	0. 60	*	0. 55	—
γ-globulin	1. 90	1. 80	*	*
Total	3. 00	1. 80	1. 05	3. 70

*Trace present and detectable but not in sufficient amount for quantitative estimation.

Results.—The data for the 5 experiments comparing the proliferation of strain L cells in fraction I (gross globulin), fraction IV (albumins), fractions I plus IV (combined in a ratio of 1:1), and in control lots of horse serum or washed serum residue are presented in text-figures 1 and 2, and are discussed in the text below. The graphs of text-figures 2 and 3 are comparable in presentation to those of text-figure 1.

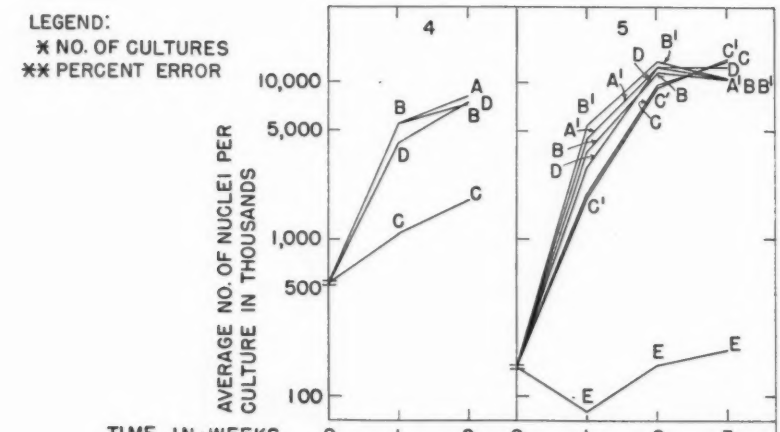
In all assays except those of experiment 5 the serum and serum fractions were tested at a protein concentration of 1.3 percent (table 1), approximately equivalent to the serum protein in a medium containing 20 percent horse serum. In experiment 5 the horse-serum residue and serum fractions were tested at a protein concentration of 2.6 percent.

In the first experiment (expt. 1, text-fig. 1) the serum fractions were prepared from fresh serum that had not been frozen. The number of cell nuclei in cultures treated with fraction I was equivalent to that in cultures treated with whole serum, whereas the cells grown in fraction IV on microscopic examination appeared to be slightly injured; many were necrotic, and the number of cell nuclei was reduced.



	*	**	*	**		*	**	*	**		*	**	*	**
A: HORSE SERUM	4	8	5	7		4	1	4	4		4	5	5	15
A^{1}: HORSE SERUM-LOT 2	–	–	–	–		–	–	–	–		4	13	5	3
B: FRACTION I (GROSS GLOBULIN)	4	10	5	10		3	10	2	4		4	9	5	5
B^{1}: FRACTION I (GROSS GLOBULIN) LOT 2	–	–	–	–		–	–	–	–		3	4	5	8
C: FRACTION IV (ALBUMIN)	4	10	4	12		4	7	5	5		–	–	–	–
D: FRACTION I + IV	–	–	–	–		4	9	5	9		3	13	2	1

TEXT-FIGURE 1.—Comparison of the effects of horse-serum fraction I (gross globulin) and fraction IV (albumins), fractions I plus IV (combined in a ratio of 1:1), and control lots of horse serum on proliferation of strain L cells. The average numbers of nuclei in the inoculum and in the cultures are plotted on a semilogarithmic grid against time in weeks. The percent error of the three inoculum samples is plotted on the graph, and the number of cultures and percent error for each treatment are indicated in the table below.



	4					5									
	*	**	*	**		*	**	*	**	*	**				
A: HORSE SERUM	4	4	5	5		–	–	–	–	–	–				
A': SERUM RESIDUE	–	–	–	–		4	11	4	3	3	3				
B: FRACTION I (GROSS GLOBULIN)	4	4	4	4		3	14	3	4	3	7				
B': FRACTION I (GROSS GLOBULIN) LOT 2	–	–	–	–		4	12	4	5	4	4				
C: FRACTION IV (ALBUMIN)	4	8	4	10		4	10	4	12	3	6				
C': FRACTION IV (ALBUMIN) LOT 2	–	–	–	–		4	14	4	7	4	7				
D: FRACTIONS I + IV	4	2	5	6		4	5	4	3	4	3				
E: SUPPLEMENTING MEDIUM	–	–	–	–		4	19	4	45	3	44				

TEXT-FIGURE 2.—The effect of horse-serum fractions and control lots of horse serum or washed serum residue on proliferation of strain L cells.

In the second experiment (expt. 2, text-fig. 1) the fractions were prepared from a pool of horse serum that had been maintained in the frozen state at −10° C. for 1 month. The number of cell nuclei in cultures treated with fraction I from this lot of serum was less than that in cultures treated with whole serum. Even when the serum components were reconstituted (fractions I plus IV), the number of cell nuclei was reduced. This finding suggested that some loss in activity or increase in toxicity occurred during the fractionation and processing of the fractions. Since fraction I had been prepared from a lot of frozen serum, it seemed desirable to test whether freezing and thawing of the serum affected the growth-promoting quality of fraction I components; therefore, in the third experiment (expt. 3, text-fig. 1), freshly drawn horse serum was divided into two lots, one of which was filtered fresh, and the other (lot 2, text-fig. 1) frozen at −10° C. for 18 hours before filtration and fractionation. The data of this experiment indicate that treatment of the cells with fraction I prepared from frozen serum resulted in as dense a population of cells as treatment with fraction I from fresh serum or as treatment with whole serum. Thus a relatively short period of freezing appeared not to affect the growth-promoting quality of fraction I components.

In the fourth experiment (expt. 4, text-fig. 2) the growth of cells in

fraction I was comparable to that of cells grown in whole serum, whereas the number of cells in cultures grown on fraction IV was still considerably lower.

In the last experiment (expt. 5, text-fig. 2) two lots of fraction I were tested—one from the same pool of freshly drawn serum as the control serum residue and the second (lot 2, text-fig. 2) from old serum that had been frozen at −10° C. for 2 months. The data indicated clearly that fraction I from 2-month-old frozen serum caused a rapid rate of cell proliferation, exceeding even that of treatment with whole serum residue; thus within these limits the age of the frozen serum appeared not to affect the quality of growth-promoting components separated in fraction I.

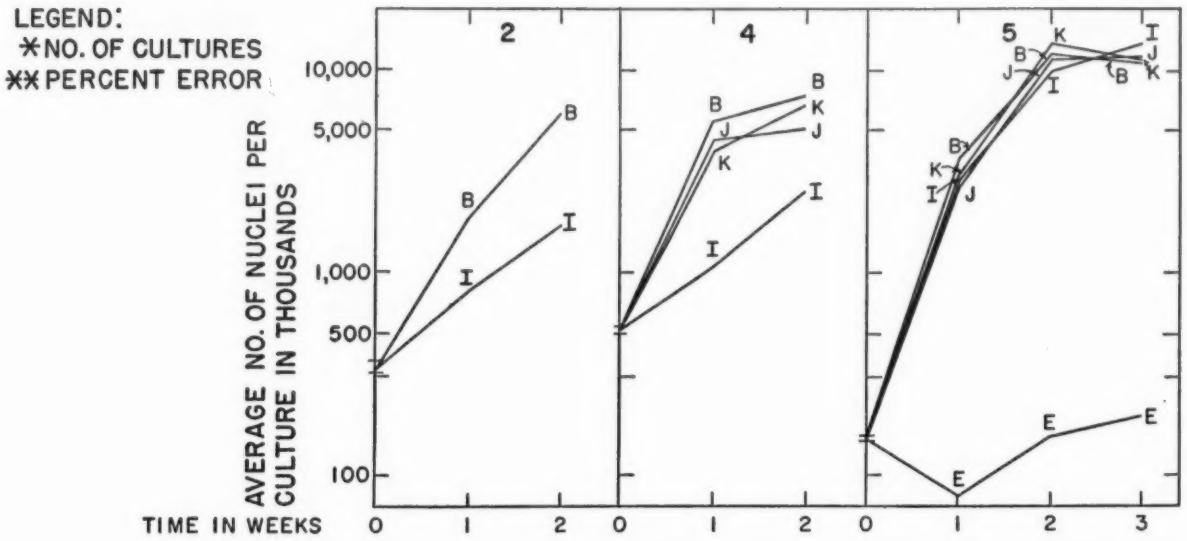
Two lots of fraction IV were also tested, one prepared by ultrafiltration to remove the alcohol and acetate and the other processed by the Cohn Method 6 and thus lacking fraction VI. There appeared to be little difference in their effects on cell proliferation. In this experiment, in which the protein concentration was twice that used previously, the number of cell nuclei in cultures treated with fraction IV was markedly increased, especially by the second and third weeks of growth. Thus at the higher concentration of protein, fraction IV appeared to be especially active in promoting cell proliferation.

The data for three experiments comparing cell proliferation in fraction II (γ-globulin), fraction III (globulin residue), fractions II plus III (combined in a ratio of 1:1), and in control lots of fraction I are presented in text-figure 3. In the first experiment (expt. 2, text-fig. 3) the fractions were tested at a protein concentration of 0.65 percent, in the second experiment (expt. 4) at a concentration of 1.3 percent, and in the last experiment (expt. 5) at a concentration of 2.6 percent.

Of the two groups of components separated from fraction I, the globulin residue (fraction III) produced a higher rate of proliferation than γ-globulin except in the last experiment when the latter was used at the higher concentration. The growth rate of cells in globulin residue appeared to be slightly less than that in fraction I. It was found that in all treatments with globulin residue, the majority of the cells failed to attach to the floor of the culture vessel; the cells grew as a dense suspension in the culture fluid, especially during the first week of growth.

Discussion

In the present study, all protein fractions isolated from horse serum were found to promote growth of strain L cells. The degree to which these fractions could be substituted for horse-serum residue without decreasing the optimal rate of cell proliferation appeared to be inversely proportional to the amount of chemical and physical manipulation necessary to separate and process the fractions; thus the γ-globulin and albumin fractions which gave the lowest proliferation rates also were exposed to potential denaturing influences (in alcohol treatment at temperatures of 5° C. and in extended ultrafiltration) to a greater degree than any of the other fractions. The gross-globulin and globulin-residue fractions, which could be sub-



	*	**	*	**		*	**	*	**		*	**	*	**	*	**
B: FRACTION I (GROSS GLOBULIN)	4	8	2	1		4	4	4	4		3	14	3	4	3	7
I: FRACTION II (GAMMA GLOBULIN)	4	17	3	31		4	9	5	3		4	20	4	5	4	8
J: FRACTION III (GLOBULIN RESIDUE)	–	–	–	–		4	4	4	9		4	20	4	4	4	5
K: FRACTION II + III	–	–	–	–		4	9	4	4		4	2	3	3	4	6
E: SUPPLEMENTING MEDIUM	–	–	–	–		–	–	–	–		4	19	4	45	3	44

TEXT-FIGURE 3.—The effect of horse-serum fractions I, II, III, and II plus III (combined in a ratio of 1:1) on proliferation of strain L cells.

stituted for the large molecular portion of horse serum with no decrease or slight decrease, respectively, in rate of cell proliferation, were not subjected to such severe treatment in the fractionation. These findings suggest that the growth-promoting component or components in horse-serum residue may be nonspecific proteins since all protein fractions tested possessed growth-promoting properties.

It is possible that the growth-promoting proteins of embryo extract most recently studied by Kutsky (*25*), the serum proteins, and other animal proteins, if purified without denaturation or chemical contamination, may be interchangeable as growth-promoting factors for tissue cells. Evans *et al.* (*10*) attempted to replace the large molecular components of horse serum with twice-crystallized, salt-free ovalbumin, which seemed to be the purest cheap protein available commercially. Ovalbumin added to a protein-free medium at a concentration of 1.3 percent increased the rate of cell proliferation, but the rate was far below that attained with horse-serum components; thus either horse serum was a much better source of protein for growth of the cells than the egg protein, or the commercial ovalbumin preparation was denatured or contaminated with traces of toxic or inhibitory material such as fatty acids or higher alcohols sometimes used for protein crystallization.

In addition to the hypothesis of a nonspecific protein growth-promoting component in the large molecular portion of horse-serum residue, it is also possible that a specific growth factor may have been present—either a protein or some nonprotein component associated with the protein fractions. If the factor were specific, the effective component would have to have been present in all fractions tested and thus present in some in very minute amounts.

An attempt was made to determine whether the ash of horse-serum residue is growth-promoting, since Healy *et al.* (*26*) had found that horse serum has a relatively large number of trace metals that have a definite effect on cell survival and multiplication. In an exploratory assay of this study no effect on cell proliferation of the ash from horse-serum residue was detected.

Since a fat-soluble vitamin might be retained in the lipoproteins of horse-serum residue, the lipids were extracted and tested. The extracted lipids when tested likewise appeared not to influence growth. This observation is in accord with that of Jacques and Barry (*27*) who, after extracting the lipids from dried human cord serum, found the extracted serum had not lost any of its activity as a growth-promoting agent when compared with unextracted dried cord serum.

There is a question as to whether the cells actually utilize the added serum protein as a source of amino acids and peptides for synthesis of cellular protein or whether the added protein plays a purely physical role in the medium. Cells fail to grow in a protein-containing medium in the absence of free amino acids (*3*) and thus appear unable to hydrolyze proteins of the surrounding medium. An effort was made in the present study to detect any decrease in the protein content of the medium during

the 48-hour interval between fluid renewals; no decrease could be detected, but with the concentration of protein used (1.3%) a decrease of over 13 mg. percent (*i.e.*, 1% of total) was necessary before being detectable by the test used. It is apparent, however, that the cells do not exhaust the gross protein supply of the medium, and yet the addition of increased amounts of protein to the medium accelerates the rate of cell proliferation (*2*). It would seem, therefore, that either the cells are utilizing constituents of the proteins without complete hydrolysis or the protein is playing some physical role in the medium. An attempt to supply such a physical factor in the form of the plasma expander dextran or the addition of methocel to a protein-free medium has so far been unsuccessful in maintaining the rate of cell proliferation obtained with the serum protein fractions (*10*).

Summary and Conclusions

An attempt has been made to isolate and identify those components of the large molecular portion of serum that appear to be essential for the rapid proliferation of strain L mouse cells *in vitro*. Horse serum has been fractionated by the Cohn low-temperature separation. When tested in a protein-free basal medium, the gross-globulin fraction could be substituted for the large molecular portion of horse serum to yield comparable rates of increase in cell numbers. After removal of the γ-globulins from the gross-globulin fraction, the residual globulins could also be substituted for the proteins of whole serum with only slight decrease in numbers of cells. A lower rate of increase in cell numbers was obtained with the albumins and γ-globulin fractions tested at a concentration of 1.3 percent; when tested at a higher concentration, however, (2.6%) the rate of increase in numbers of cells grown in γ-globulins was the same and in the albumins was less than that of cells grown in the same concentration of the unfractionated serum proteins.

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